TAMPERE UNIVERSITY OF TECHNOLOGY

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ATTACHMENT AND DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELL DERIVED RETINAL PIGMENT EPITHELIUM CELLS ON DIFFERENT COATING MATERIALS

Master of Science Thesis

Examiners: professors Minna Kellomäki and Heli Skottman
Examiners and topic approved in the Faculty of Science and Environmental Engineering Council meeting on April 7th, 2010

ABSTRACT

TAMPERE UNIVERSITY OF TECHNOLOGY
Master's Degree Programme in Biotechnology
SAVIOJA, ELINA: Attachment and differentiation of human embryonic stem cell derived retinal pigment epithelium cells on different coating materials
Master of Science Thesis, 77 pages
December 2010
Major: Tissue engineering
Examiners: Professors Minna Kellomäki and Heli Skottman
Keywords: Human embryonic stem cell, retinal pigment epithelium cell, coating material, attachment, differentiation

Age-related macular degeneration (AMD) and some other retinal diseases are associated with degeneration of retinal pigment epithelium (RPE) cells. A potential cure could involve cell transplantation therapy using RPE cells differentiated from pluripotent human embryonic stem cells (hESCs). A big challenge is to produce a homogeneous population of hESC-derived RPE (hESC-RPE) cells, and coating materials are one potential alternative to promote the differentiation.

The aim of this study was to evaluate the cell attachment and differentiation of hESC-RPE cells on different coating materials and culture conditions. hESCs were derived and maintained without serum and animal feeder cells. This study consisted of three parts. In the first part, hESCs were cultured on different xeno-free coating materials and in a culture medium that stimulated the differentiation towards RPE cells. CELLstart™, collagen IV, and VitroCol™ were the best coating materials to support the cell attachment. The differentiation of the hESCs towards RPE cells was analyzed by visual pigment observation, immunocytochemistry, and quantitative real time PCR. There were no significant differences in the differentiation of the cells cultured on CELLstart™, collagen IV, or VitroCol™.

In the second part, confocal microscopy wells were tested. hESCs differentiating to RPE cells and enzymatically degraded hESC-RPE cells attached and grew best in collagen IV coated Ibidi wells. In the third part, hESC-RPE cells were cultured in BD BioCoat™ collagen IV cell culture inserts in culture mediums containing basic fibroblast growth factor (bFGF) or not. RPE cells attached and grew well in the inserts with or without bFGF.

The good attachment of hESCs differentiating towards RPE cells to CELLstart™, collagen IV, and VitroCol™ coatings was a remarkable result, since to my knowledge no publication of hESC differentiation towards RPE cells with a xeno-free coating material and a serum-free culture medium in adherent cell culture exists. Collagen IV can be recommended as a coating material for hESCs differentiating towards RPE cells, since it is cheaper than CELLstart™ and VitroCol™ and easy to use. In the future, the effects of growth factors and other supplements on RPE differentiation could be studied. Collagen IV can also be recommended for Ibidi confocal microscopy wells for hESC-RPE cells, at least for short-term experiments. Longer-term effects should be further studied. BD BioCoat™ collagen IV cell culture inserts can be utilized in research purposes, but they are not optimal since they contain xeno-products. In the future, the ability of bFGF to promote RPE cell cluster distribution and to transdifferentiate RPE cells could be further studied.

TIIVISTELMÄ

TAMPEREEN TEKNILLINEN YLIOPISTO
Biotekniikan koulutusohjelma
SAVIOJA, ELINA: Ihmisalkion kantasoluista erilaistettujen verkkokalvon pigmenttisolujen kiinnittyminen ja erilaistuminen erilaisilla pinnoitemateriaaleilla
Diplomityö, 77 sivua
Joulukuu 2010
Pääaine: Kudosteknologia
Tarkastajat: Professorit Minna Kellomäki ja Heli Skottman
Avainsanat: Ihmisalkion kantasolu, verkkokalvon pigmenttisolu, pinnoitemateriaali, kiinnittyminen, erilaistuminen

Silmänpohjan rappeumaan ja joihinkin muihinkin verkkokalvon sairauksiin liittyy verkkokalvon pigmenttisolujen (RPE-solujen) rappeutuminen. Mahdollinen hoito näihin sairauksiin voisi olla soluterapia, jossa käytettäisiin ihmisalkion kantasoluista (hES-soluista) erilaistettuja RPE-soluja, sillä hES-soluilla on kyky muodostaa kaikkia aikuisen yksilön kudoksia. Suurena haasteena on tuottaa homogeeninen solupopulaatio hES-soluista erilaistettuja RPE-soluja (hESC-RPE -soluja) ja pinnoitemateriaalit ovat yksi potentiaalinen vaihtoehto edistämään erilaistumista.

Tässä tutkimuksessa arvioitiin hESC-RPE -solujen kiinnittymistä ja erilaistumista erilaisilla pinnoitemateriaaleilla ja viljelyolosuhteissa. hES-solujen eristyksessä tai kasvatuksessa ei käytetty seerumia tai eläinperäisiä tukisoluja. Kolmiosaisen tutkimuksen ensimmäisessä osassa hES-soluja viljeltiin erilaisilla eläinvapailla pinnoitemateriaaleilla sekä soluviljelynesteessä, joka stimuloi erilaistumista RPE-soluiksi. CELLstart™, kollageeni IV ja VitroCol™ pinnoitteet edistivät parhaiten solujen kiinnittymistä. hES-solujen erilaistumista RPE-soluiksi analysoitiin tarkkailemalla pigmentoitumista, immunosytokemialla ja kvantitatiivisella reaaliaikaisella PCR:llä. CELLstart™, kollageeni IV ja VitroCol™ pinnoitteiden päällä kasvaneiden solujen erilaistumisissa ei ollut merkittäviä eroja.

Toisessa osassa testattiin konfokaalimikroskooppikuoppia. RPE-soluiksi erilaistuvat hES-solut sekä entsymaattisesti hajotetut hESC-RPE -solut kiinnittyivät ja kasvoivat parhaiten kollageeni IV Ibidi -kuopilla. Kolmannessa osassa hES-soluista erilaistettuja RPE-soluja viljeltiin BD BioCoat™ kollageeni IV -inserteissä ja soluviljelynesteissä, joista osa sisälsi fibroblastista kasvutekijää (bFGF). RPE-solut kiinnittyivät ja kasvoivat hyvin inserteissä riippumatta siitä, oliko soluviljelynesteessä bFGF:ää vai ei.

RPE-soluiksi erilaistuvien hES-solujen hyvä kiinnittyminen CELLstart™, kollageeni IV ja VitroCol™ pinnoitteisiin oli merkittävä tulos, sillä minun tietääkseni tähän mennessä ei ole julkaistu tutkimusta, jossa hES-solut olisivat erilaistuksessa RPE-soluiksi kiinnittyneet eläinvapaisiin pinnoitteisiin seerumivapaissa olosuhteissa. Kollageeni IV:ää voidaan suositella pinnoitemateriaaliksi RPE-soluiksi erilaistuville hES-soluille, koska se on halvempi kuin CELLstart™ ja VitroCol™ sekä helppokäyttöinen. Tulevaisuudessa voisi tutkia kasvutekijöiden ja muiden lisäaineiden vaikutuksia RPE-solujen erilaistumiseen. Kollageeni IV:ää voidaan suositella myös Ibidi -konfokaalimikroskooppikuoppiin hESC-RPE -soluille, ainakin lyhytaikaisesti. Pitkäaikaisempia vaikutuksia pitää kuitenkin tutkia lisää. BD BioCoat™ kollageeni IV -inserttejä voidaan käyttää tutkimustarkoituksissa, mutta ne eivät ole ihanteellisia niiden sisältämien eläinperäisten aineiden takia. Tulevaisuudessa bFGF:n kykyä edistää RPE-solurykelmien hajotusta ja RPE-solujen erilaistumista uudelleen muiksi soluiksi voisi tutkia tarkemmin.

PREFACE

This study was carried out at Regea - Institute for Regenerative Medicine, University of Tampere in Ophthalmology Group.

First, I would like to thank my supervisors. I owe my gratitude to Prof. Heli Skottman for supervising me throughout this thesis. I acknowledge Prof. Minna Kellomäki for her valuable guidance especially at the end of this project.

I would like to express my sincere gratitude to Hanna Vaajasaari, M.Sc, for helping and advising me in the practical work. This study would have not been possible without Hanna. I am grateful to Ms. Elina Konsén and Ms. Hanna Koskenaho for helping me in the laboratory work. Also, I want to thank the whole personnel of Regea for supportive working environment.

Finally, I would like to express my gratitude to my family and friends for supporting me throughout my studies. Above all, I am grateful to Hannu for his love, encouragement and advice.

Tampere, 24.10.2010

Elina Savioja

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ABBREVIATIONS AND NOTATIONS

2D	Two-dimensional
3D	Three-dimensional
AMD	Age-related macular degeneration
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHX10	Ceh-10 homeodomain containing homolog
CRALBP	Cellular retinaldehyde-binding protein
DAPI	4´, 6´-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
EB	Embryoid body
ECM	Extracellular matrix
FBS	Fetal bovine serum
FRET	Förster Resonance Energy Transfer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMP	Good manufacturing practice
hESC	Human embryonic stem cell
hESC-RPE	Human embryonic stem cell derived retinal pigment epithelium cell
hFF	Human foreskin fibroblast
ICM	Inner cell mass
iPSC	Induced pluripotent stem cell
IVF	*In vitro* fertilization
Ko-SR	Knockout™ Serum Replacement
MEF	Mouse embryonic fibroblast
MITF	Microphthalmia-associated transcription factor
mRNA	Message RNA
NDS	Normal donkey serum
OCT-4	Octamer-binding transcription factor
OTX2	Orthodenticle homeobox 2
PA6 cells	Stromal cell line derived from bone marrow
PAX6	Paired box gene
PCR	Polymerase chain reaction
PB	Phosphate buffer
PEI	Polyethyleneimine
POS	Photoreceptor outer segment
q-RT-PCR	Quantitative real time polymerase chain reaction
RAX	Retina and anterior neural fold homeobox
RNA	Ribonucleic acid

RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RPE DM- medium	Culture medium used to induce the differentiation of hESCs towards RPE cells
RPE65	Retinal pigment epithelium-specific protein 65 kDa
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SSEA	Stage-specific embryonic antigen
SIX3	Sine oculis homeobox homolog 3
TCEP	Tris(2-carboxyethyl)phosphine
TRA	Tumor-related antigen
VEFG	Vascular endothelial growth factor
Xeno-free	Free from animal-derived material
ZO-1	Zonula occludens-1

1. INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent cells which can differentiate into all cell types of the human body, and therefore are considered as a valuable source for cell transplantation and tissue engineering. Several diseases, including Parkinson's disease, diabetes, and spinal cord injury, result from the loss of a specific cell type in the tissue. The replacement of those lost cells with hESC-derived cells could cure these and many other degenerative diseases. Among these diseases are age-related macular degeneration (AMD), Best disease, and subtypes of retinitis pigmentosa (RP). These conditions are associated with progressive degeneration of retinal pigment epithelium (RPE) cells, leading often to blindness. A potential cure could involve cell transplantation therapy using RPE cells differentiated from hESCs.

To date, hESCs have been successfully differentiated into several different cell types, and various differentiation methods of hESCs towards RPE cells have been published (Klimanskaya et al. 2004; Klimanskaya 2006; Lund et al. 2006; Gong et al. 2008; Osakada et al. 2008; Vugler et al. 2008; Idelson et al. 2009; Lu et al. 2009; Nistor et al. 2010). One of the main challenges in the differentiation is the development of a homogeneous population of RPE cells. There are still many unsolved challenges in the differentiation of hESCs, and comparatively little is known about the molecular mechanisms involved in the differentiation of the hESCs into specific cell types. Nevertheless, the cell population has to be homogeneous for therapeutic applications. Various methods have been tested to promote the differentiation, and coating materials are one potential alternative. Cell-material interactions are complicated and affect cell properties. However, it seems that these interactions on two-dimensional (2D) -coating materials have not been so largely studied than on three-dimensional (3D) -implants. Advantages of the coating materials are that many of them are xeno-free and composed of proteins found in the human body. They could replace largely used mouse embryonic fibroblasts (MEFs), and therefore provide xeno-free culture conditions. Culture medium should also be optimized and xeno-free. In adherent cell culture the crucial factor is whether the coating material supports the cell attachment or not. If the coating material supports the attachment, its ability to support the differentiation can be studied next. Several studies, including preceding ones, utilize already coating materials in hESC-derived RPE (hESC-RPE) cell differentiation protocols.

Nevertheless, to my knowledge no study of hESC differentiation towards RPE cells with a xeno-free coating material and a serum-free culture medium in adherent cell culture has been published. In most studies, the differentiation is stimulated in embryoid bodies (EBs). Few publications (Klimanskaya et al. 2004; Klimanskaya 2006; Lund et

al. 2006) have used adherent culture conditions in the differentiation of hESCs towards RPE cells, but the media in those studies have contained fetal bovine serum (FBS). FBS is used to improve the cell attachment, but the use of it is controversial due to ethical and scientific reasons.

The aim of this thesis is to evaluate the cell attachment and differentiation of hESC-RPE cells on different coating materials and culture conditions. Human RPE cells, hESCs, and coating materials are discussed in the section of Theoretical background. Next, the experimental study, which consists of three parts, is presented. The aim of the first part is to evaluate the effects of xeno-free coating materials on the attachment and differentiation of hESCs differentiating towards RPE cells. The aim of the second part is to evaluate the attachment and growth of hESC-RPE cells to Ibidi confocal microscopy wells. The aim of the third part is to evaluate the attachment and growth of mature hESC-RPE cells in BioCoat™ collagen IV cell culture inserts. In all the experiments, hESCs are derived and maintained without serum and animal feeder cells. Finally, conclusions will be made, and recommendations for the future will be given.

2. THEORETICAL BACKGROUND

2.1. Retinal pigment epithelium (RPE)

2.1.1. Structure of the eye

The human eyeball is globe-shaped with a diameter of approximately 2.5 cm, and it is located in an eye socket. The basic structure of the eye is presented in Figure 2.1. Eye wall consists of three layers. The outer layer is a strong and tough sclera. In the front part of the eye, the sclera transforms to a transparent cornea which allows light to enter the eyeball. The middle layer is a thin and highly vascular choroid. It contains multiple pigment cells that prevent the reflections coming from inside the eye. Forward the eye, the choroid extends to form an iris that surrounds the pupil. The amount of the pigment in the iris defines individual's eye color. Smooth muscle fibers in the iris form pupil dilator and constrictor muscles. By changing the diameter of the pupil, the amount of the light reaching the retina can be regulated. (Haug et al. 1995.)

Between the iris and the choroid is located a ciliary body which is a muscle formed of smooth muscle fibers. A transparent and elastic lens is attached to the eye with thin filaments that connect the ciliary body and the surface of the lens. The lens divides the eye into an anterior chamber and a large gelatinous vitreous body. The small anterior chamber is filled with aqueous humor which transports nutrients to the cornea and the lens, and waste products out. (Haug et al. 1995.)

The inner layer of the eye is a retina, where the light entering the eye is focused. The structure of the retina is discussed in the following chapter. In the back of the eye there is an optic nerve which delivers electric signals to the brain. The modification of an image starts at the retina, but the eventual picture formation takes place in the brain. (Haug et al. 1995.)

Movable eyelids protect the eye from strong light, mechanical damage, and endangering particles. Eyelid movements smear tear fluid to the outer surface of the eye. The tear fluid moisturizes and sanitizes the outer surface. In addition, it contains enzymes that inhibit bacterial growth. (Haug et al. 1995.)

2.1.2. Structure of the retina

The innermost layer of the eyeball, the retina, consists of two parts. Neural retina is the inner layer and pigment epithelium the outer layer. The neural retina can be further subdivided into its various cell layers with different functions. For instance, ganglion, amacrine, bipolar, and horizontal cells are located in the neural retina (Figure 2.1). Light, which enters optic fiber layer side first, must penetrate all cell types before

reaching photoreceptors, rods, and cones. (WebVision 2003; Drake et al. 2005.) The cones are densely concentrated in the area called macula which therefore has the highest visual acuity of the retina (Ehrlich et al. 2008).

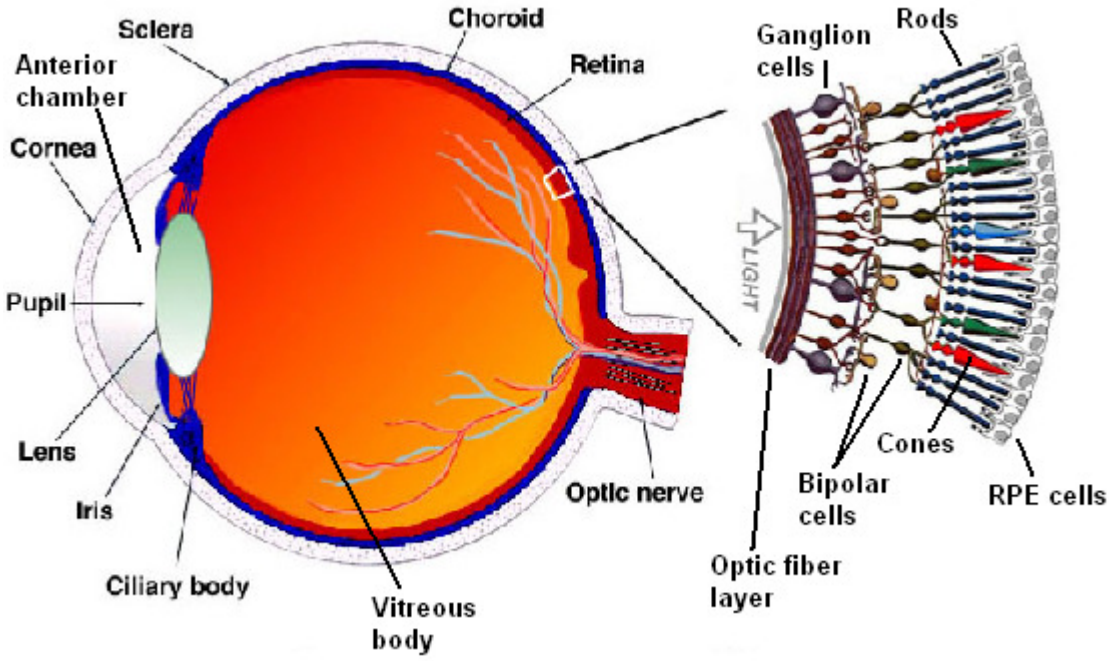

Figure 2.1. *The structure of the eye and the retina (modified from WebVision 2003).*

The neural layer attaches to RPE layer. RPE cells are discussed in the following chapter. Between the RPE layer and the choroid is located five-layered Bruch's membrane which participates in maintaining photoreceptor viability as well as overall retinal health. It is involved in the crucial exchange of several biomolecules, oxygen, nutrients, and waste products. Furthermore, it plays a significant role in the cell-cell communication, cell differentiation, proliferation, and migration. The nature of the Bruch's membrane is highly dynamic. (Booij et al. 2009.)

2.1.3. Retinal pigment epithelium cells

RPE is a derivative of a neuroectoderm which is the portion of an early embryo ectoderm that gives rise to central and peripheral nervous systems, and therefore RPE cells share the same progenitors with the neural retina in early development. The RPE is composed of a continuous monolayer of simple cuboidal pigmented cells located between the neural retina and the choroid (Lu et al. 2001; Strauss 2005). Normally the RPE cells are hexagonal-shaped and packed together resembling cobblestones (Figure 2.2). The size of an RPE cell varies depending on the location in the retina and on person's age. For young people, the height of a cell in a macula is approximately 14 µm and the width 10-14 µm. Melanin and other pigments give the RPE a mottled brown color. The RPE cells have a complex structural and functional polarity, which enables them to perform highly specified roles. There are distinct apical, basal, and lateral

surfaces in the RPE cell membrane. The apical surface is covered with microvilli. The basal surface is twisted into several basal infoldings, enabling effective molecular transport. The lateral surfaces of the adjacent RPE cells are joined together by four junction types: tight junctions, adherent junctions, desmosomes, and gap junctions. (Lu et al. 2001.)

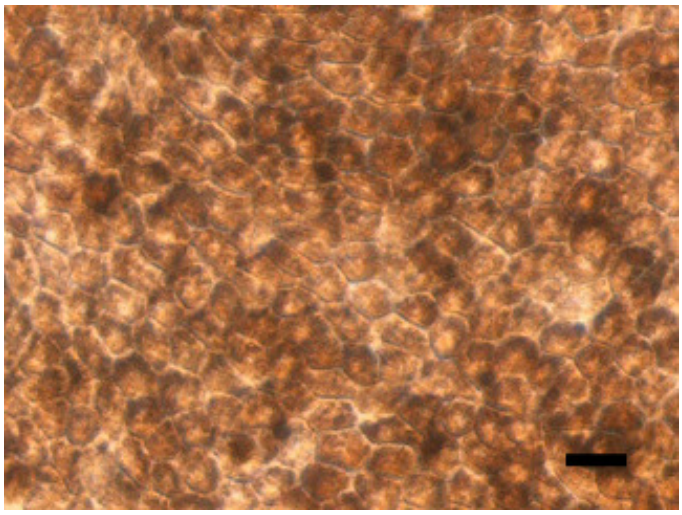

Figure 2.2. *Cell culture of hESC-RPE cells (unpublished). Scale bar 20 μm.*

The RPE provides crucial support for the long-term preservation of the retinal integrity and visual function (Carr et al. 2009). The RPE cells participate in many processes critical for photoreceptor survival, including nutrient and ion transport, and recycling of the retina (Strauss 2005). They contain melanin granules that play a significant role in protecting the retina from the damage caused by light (Ming & Le 2007). The functions of the RPE cells include also transport and processing of vitamin A (Marmorstein et al. 1998), the regeneration of retinoids, and tissue repair (Klimanskaya et al. 2004). In addition, they form part of the blood-retinal barrier via their tight junctions, and are involved in retinoid metabolism by generating 11-*cis*-retinaldehyde (Ming & Le 2007).

The very important property of the RPE cells is the phagocytosis of photoreceptor outer segments (POSs) which is a significant process for the renewal of photoreceptor membranes (Carr et al. 2009). The RPE cells *in vivo* differ from many phagocytic cells because they normally ingest only one type of particle: the POSs. However, in cell culture, the RPE cells can also bind and ingest a variety of substances, including latex beads (Klimanskaya et al. 2004; Osakada et al. 2008), though they preferentially phagocytose the POSs. (reviewed by Carr et al. 2009.)

An extraordinary character of the RPE is its obvious plasticity. Normally the RPE cells are mitotically quiescent, but they begin to divide in response to an injury or photocoagulation. The RPE cells next to the injury become flatter and proliferate forming a new monolayer. Various studies have indicated that the RPE monolayer can produce cells of fibroblast appearance which can later return to their original RPE morphology. (Klimanskaya et al. 2004.)

2.1.4. Disorders of the RPE

Malfunctions of the RPE cells can disturb the visual perception by affecting photoreceptors, which eventually lead to different retinal diseases. Dysfunction, degeneration, and loss of the RPE cells are prominent features of for instance AMD, RP, and Best disease (Idelson et al. 2009).

AMD is the leading cause of blindness among people over 60 years in the western world (Carr et al. 2009). Worldwide, the AMD is the third cause of blindness (Katta et al. 2009), and it accounts for 16, 000 new cases of severe loss of vision annually. According to its name, the AMD means the degeneration of the macula, and therefore central vision loss may occur. The AMD can exhibit various morphologic forms. The most common condition is a dry, atrophic type, which starts with a progressive loss of central vision in one eye and spreading to the other. In the atrophic AMD, the RPE cells become atrophied and less pigmented. They are also missing in places, and stacked up in others. Choroid is degenerated, and deposits form between the RPE layer and Bruch's membrane leading to distortion. A wet, exudative type has the same pathogenesis as the atrophic type, but it is also characterized by choroidal neovascular growth under the RPE and the retina with subsequent hemorrhage. The exudative AMD can lead to a rapid loss of central vision since the hemorrhage does not resorb, and fibrovascular tissue invades from the choroid and retinal blood vessels. (Lu et al. 2001; Zarbin 2004.) The factors causing the AMD are believed to be both genetic disorders and environmental factors, including age, obesity, and smoking. Current treatment modalities in the AMD are a dietary supplementation of anti-oxidants, laser therapy, anti-vascular endothelial growth factor (VEFG), and a combination therapy of laser with anti-VEFG treatment, but complete reversal of the degeneration is not possible. (Katta et al. 2009.)

RP is a group of inherited disorders in which the abnormalities of photoreceptors or RPE cells lead to a progressive visual loss (Pagon & Daiger 2005). Prevalence of this eye disease is approximately 1 in 5000 worldwide. The age of onset can vary, but the RP is usually diagnosed in young adulthood. (Telander et al. 2009.) Patients with the RP experience first defective dark adaptation followed by the constriction of the peripheral field, and finally the loss of central vision (Pagon & Daiger 2005). In this final pathway, rod photoreceptors are usually killed by apoptosis, which leads to the vision loss (Telander et al. 2009). The RP is diagnosed by the documentation of progressive loss in photoreceptor function by electroretinography and visual field testing. The inheritance mode of the RP is determined by family history. At least 35 different genes or loci are known to cause nonsyndromic RP. (Pagon & Daiger 2005.) The RP has significant phenotypic variations since there are various genes that can lead to a diagnosis of the RP, and patients with the same genetic mutation can have very different retinal findings (Telander et al. 2009). The RP can be inherited in an autosomal recessive, autosomal dominant, or X-linked manner. Some mitochondrial and digenic forms have also been

reported. A therapy with vitamin A palmitate may slow retinal degeneration in the RP, but so far there is no remedy. (Pagon & Daiger 2005.)

Best disease, also called vitelliform macular dystrophy, is a slowly progressive macular dystrophy with onset usually in childhood and sometimes in later teenage age (MacDonald & Lee 2009). This rare eye disease results from a disorder in the RPE. (Altaweel 2010). Patients with the Best disease initially have normal vision followed by metamorphopsia and central visual acuity. Nevertheless, the patients retain normal peripheral vision and dark adaptation. The diagnosis of the Best disease is based on fundus appearance, electrooculogram, and family history. The patients have a typical yellow yolk-like lesion in the macula. (MacDonald & Lee 2009.) The Best disease is inherited in an autosomal dominant manner and no treatment exits for the moment (Altaweel 2010). Other retinal dystrophies caused by RPE degeneration are Stargardt's disease, pattern dystrophies, choroideremia, and photic maculopathy (Lu et al. 2001).

RPE dysfunctions, including AMD, RP, and Best disease are connected to progressive visual loss leading often to blindness. Several therapeutic approaches to delay the degenerative process are under development, but for the meantime many patients ultimately lose their sight. Cell therapy to replenish the degenerating RPE cells is one alternative to halt disease progression. Lost cells could be replaced with stem cell derived cells.

2.2. Stem cells

Stem cells are cells which are capable of renewing themselves, and can differentiate into many different cell types. They can divide either symmetrically producing two new daughter cells or asymmetrically producing one daughter stem cell and one differentiating cell. Human stem cells can be found in embryo, fetus, amniotic fluid (De Coppi et al. 2007), and in some adult tissues, for instance in skin, bone marrow, skeletal muscle, brain, and heart. Researchers have also discovered another type of stem cells, induced pluripotent stem cells (iPSCs), which are largely studied. iPSCs are pluripotent cells derived from any differentiated cell type through an ectopic expression of transcription factors (Amabile & Meissner 2009).

Stem cells possess a hierarchical differentiation potential according to their origin (Figure 2.3). The most potential cells are called totipotent stem cells which are capable of developing into a complete organism or differentiating into any of its cells or tissues. Totipotent stem cells are found in dividing zygote and early blastomeres (Amabile & Meissner 2009). Differentiation of the totipotent stem cells results in the formation of a blastocyst which consists of two primary cell lines: an undifferentiated inner cell mass (ICM) and an outer layer of trophoblast cells. The ICM contains pluripotent stem cells which are capable of differentiating all germ layers, endoderm, mesoderm, and ectoderm, and also the germline, but not extra embryonic tissues (Amabile & Meissner 2009). Adult stem cells are multipotent cells which can differentiate into multiple cell types, but within a certain lineage. Multipotent stem cells specialize further to

progenitor cells which can produce only one type of cells, but have the capability to self-renew. (Stem Cell MLM 2010.)

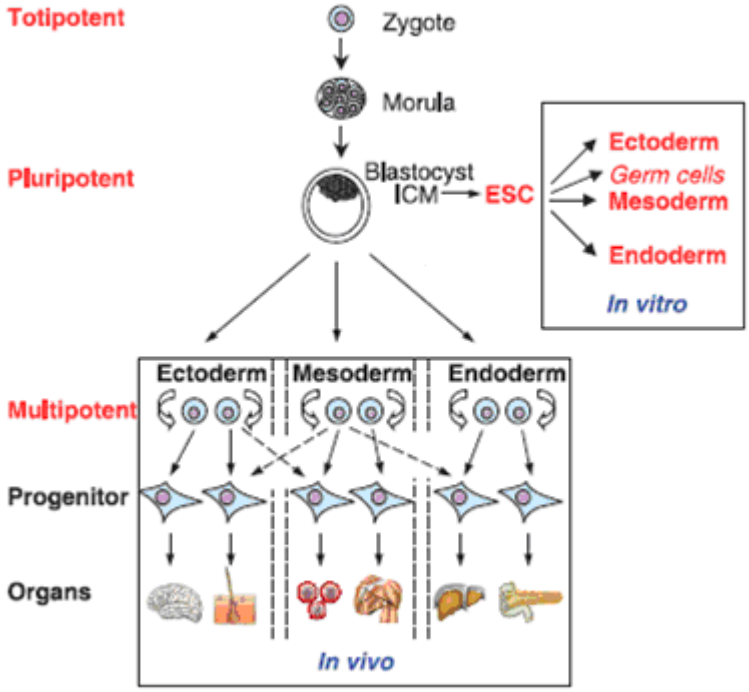

Figure 2.3. *Human stem cell hierarchy (modified from Wobus & Boheler 2005).*

2.3. Human embryonic stem cells (hESCs)

The first stable hESC lines were derived in the USA by Thomson and co-workers (1998). In the present-day culture protocol, hESCs used in cell culture are usually isolated from the ICM of a four to five day old embryo. These embryos are usually poor quality embryos from *in vitro* fertilization (IVF) treatment laboratories (Liu et al. 2009).

At the blastocyst stage, a hollow sphere of cells is formed which contains an outer cell layer and an inner cluster of cells called ICM. The outer cells become the trophoectoderm, and subsequently give rise to the placenta and other supporting tissues, whereas the ICM will finally create all tissues in the body. (Winkel & Pederson 1988.) The isolation of the ICM cells usually involves the use of enzymes and immunosurgery where the trophoectoderm is selectively removed using rabbit anti-human antibodies. After the isolation, the cells of the ICM are propagated on diverse types of feeder cells or on suitable extracellular matrix (ECM) under feeder-free conditions. (Skottman et al. 2006.) ECM is a complex structural entity that surrounds and supports the cells in the tissues. ECM is composed of fibrous proteins and glycosaminoglycans, most abundant proteins being collagen and elastin. Other proteins in ECM are for instance fibronectin, vitronectin, laminin, and tenascin. (Rozario & DeSimone 2010.) The ICM isolation and hESC derivation are presented in Figure 2.4.

hESCs are capable of self-renewal because they have a prolonged replication capacity due to high levels of telomerase activity (Thomson et al. 1998; Amit et al.

2000). Another main characteristic of the hESCs is pluripotency. Thus, they can differentiate *in vitro* and *in vivo* into a wide variety of cell types, like the derivatives of three embryonic germ layers which will finally give rise to all the tissues and organs of the body. Furthermore, the hESCs have high alkaline phosphatase activity (Thomson et al. 1998). The differentiation potential of the hESCs has raised hope that these cells could be a renewable source for cell transplantation in severe degenerative diseases (Hoffman & Carpenter 2005).

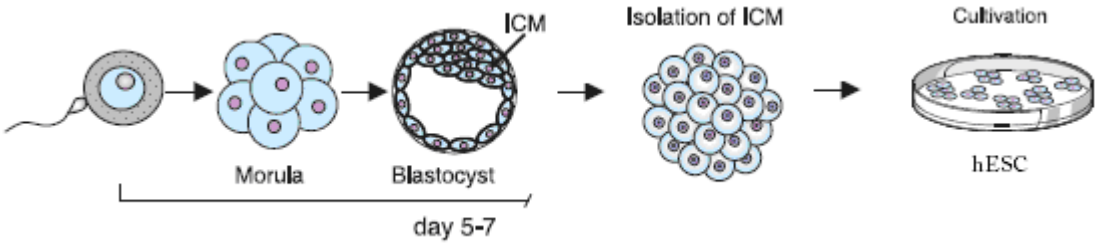

Figure 2.4. *Human pluripotent stem cells can be derived from in vitro cultured ICM cells of the blastocyst after IVF (modified from Wobus & Boheler 2005).*

2.3.1. Culturing of hESCs

Culturing of hESCs is a very time-consuming process. After the hESCs have been isolated from the ICM, they start to form colonies, and might spontaneously differentiate. To remain in the undifferentiated state, the hESCs need to be passaged onto new culture, culture medium has to be regularly changed and morphology frequently observed. The culture medium composition, decision of using feeder cells or not, and passages are crucial elements in hESC culture conditions.

Culture medium contains nutrients for the cells, and possibly other supplements to control the growth and differentiation. The culture medium of the hESCs is supposed to maintain the pluripotency and self-renewal capability of the cells. Culturing of the hESCs requires daily media changes. Earlier, FBS and human serum were largely used in hESC culture media. FBS is a complex mixture of different factors which promote cell adhesion and growth (Klauser et al. 2010). It contains for instance growth factors, proteins, vitamins, trace elements, and hormones (van der Valk et al. 2010). Nevertheless, the use of FBS is controversial due to ethical and scientific reasons. The collection of serum causes needless suffering for unborn calves. In addition, the variance between different FBS batches is normally relatively high because it is gained from a living organism (Klauser et al. 2010; van der Valk et al. 2010.) This causes phenotypical differences in the cell cultures, and thus variations of the results (van der Valk et al. 2010). Different batches vary also in their capability of maintaining hESCs at an undifferentiated stage. Several research groups have optimized serum-free culture medium containing serum replacement (Knockout™ Serum Replacement, Ko-SR, Gibco, Invitrogen). (Unger et al. 2008.) Ko-SR with basic fibroblast growth factor (bFGF) is widely used. bFGF is thought to help to retain the self-renewal of hESCs (Trounson 2006). Even though Ko-SR provides better defined culture conditions

compared to the serum-containing medium, it contains animal-derived AlbuMAX, and therefore it is not optimal either. Recent hESC culture techniques utilize also various ECM components, activin A (Skottman et al. 2006), bone morphogenetic proteins signaling antagonist Noggin, nicotinamide, and keratinocyte growth factor. The use of different supplements depends on whether culture conditions contain feeder cells or not. (Unger et al. 2008.) The future challenge is to produce a xeno-free culture medium for hESC culture.

hESCs are commonly cultured on mitotically inactivated feeder cells. The mitotic inactivation can be done by irradiation, and due to it, feeder cells cannot replicate, but still secrete proteins and other factors that maintain the undifferentiated growth of the hESCs. Feeder cells provide also applicable cell-cell contacts for the attachment of the hESCs. MEFs have been widely used as feeder cells in hESC culture, but so far several human cell lines have been tested as feeder cells for the hESCs to replace the animal cells. Various human cell types have supported the hESC growth and even their derivation. Fibroblasts supporting the hESCs have been derived from the muscle of aborted embryos, fetal skin, and from adult tissues, including foreskin and fallopian tube. In addition, human marrow-derived stromal cells, cells from uterine endometrium, and even hESC-derived fibroblasts have been tested as feeders. (reviewed by Unger et al. 2008.)

The most optimal culture condition for hESCs would be a feeder-free and fully-defined system, but that is still under development. Various coating materials with supplements in the culture medium have been tested in order to find a combination that would mimic the supportive impact of the feeder cells. Klimanskaya and co-workers reported the derivation of the first feeder-free hESC lines using a mouse-derived matrix, but such matrix is not probably optimal for clinical use (Klimanskaya et al. 2005). Ludwig and co-workers have derived two new hESC lines on Matrigel™ and on human laminin in a chemically defined medium, but some genetic abnormalities appeared (Ludwig et al. 2006). It is possible that feeder-free cultures are so demanding for the hESCs that the cells become more susceptible to genetic alterations (Draper et al. 2004). Matrigel™ is an ECM extract from mouse sarcoma with major component laminin, followed by collagen IV, heparan sulphate proteoglycans, and nidogen. It contains also growth factors and tissue plasminogen activator. (Gong et al. 2008.) Due to animal origin, Matrigel™ does not provide optimal culture conditions for hESCs.

hESCs need to be passaged onto new culture regularly. Multicellular colonies of the hESCs are dispersed to smaller cell clusters mechanically or enzymatically. In the mechanical passaging, undifferentiated parts of hESC colonies are cut into small pieces with a scalpel, and detached from the feeders with a needle. hESC colonies are judged as undifferentiated and differentiated parts by the morphology. Mechanical passaging is laborious and time-consuming, but it does not require additional substances. Enzymatic passaging is a more rapid and straightforward technique, in which the cells are dispersed for instance with trypsin, dispase, or collagenase. However, enzymatic passaging is not optimal for the cells since repetitive exposure to enzymes may cause genetic

abnormalities (Buzzard et al. 2004; Mitalipova et al. 2005). Furthermore, enzymatic passaging results in variable-sized cell clusters, and the exclusion of differentiated parts is difficult. Used enzymes are also often xeno-products. Whether the mechanical or enzymatic passage method is used, the hESCs are moved onto new feeder cells or on suitable ECM under feeder-free conditions every five to ten days depending on the hESC line.

2.3.2. Characterization of hESCs

hESC lines should be characterized regularly to ascertain that specific characters and stable karyotypes are maintained during the expansion (Hoffman & Carpenter 2005). At first, hESCs are typically judged as undifferentiated by their morphology. Undifferentiated hESCs have a high ratio of nucleus to cytoplasm and prominent nucleoli (Thomson et al. 1998). On feeder cells, the undifferentiated hESCs form dense and even colonies with sharp edges (Figure 2.5). The morphology of the hESCs cultured on feeder-free conditions may differ from that of the hESCs cultured on a feeder cell layer. The specific morphology of pluripotent hESCs is a functional and widely used method of observing the growth of the undifferentiated hESCs.

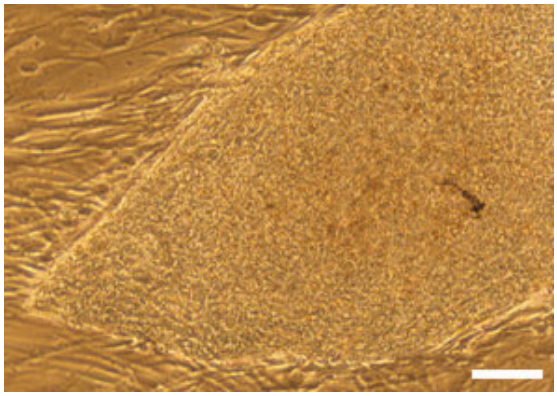

Figure 2.5. *Typical morphology of a hESC colony on feeder cells (modified from Rajala et al. 2007). Scale bar 200 µm.*

A more accurate characterization is done by analyzing the expressions of different markers and genes, typical for undifferentiated and differentiated hESCs. Undifferentiated hESCs express cell surface markers such as stage-specific embryonic antigens (SSEA-3 and SSEA-4) and tumor-related antigens (TRA-1-60 and TRA-1-81), whereas differentiated hESCs express SSEA-1 (Thomson et al. 1998). The undifferentiated hESCs express also alkaline phosphatise, and transcription factors octamer-binding transcription factor (OCT-4) and Nanog (Thomson et al. 1998; Reubinoff et al. 2000; Hart et al. 2004).

The pluripotency of the hESCs can be characterized after the derivation of the hESC line by teratoma formation in mouse. hESCs are injected into severed combined immunodeficient mice in which the hESCs form a teratoma composing of cells from all three germ layers: endoderm, mesoderm, and ectoderm. Resulting teratomas are

examined histologically. (Thomson et al. 1998.) Another method of exploring the pluripotency of the hESCs is EB formation. hESCs are cultured in suspension without feeder cells and with a medium lacking bFGF. In these culture conditions, the hESCs spontaneously aggregate and form EBs where they differentiate into derivates of all three germ layers. Different cell types can be recognized by isolating ribonucleic acid (RNA) from the EBs and using reverse transcription polymerase chain reaction (RT-PCR) with primers for typical genes for different germ layers. (Itskovitz-Eldor et al. 2000.)

2.3.3. Ethical issues and regulations concerning hESC research

Although hESCs possess a great promise for the future regenerative therapies, the research area is also considered as a difficult ethical issue. The ethical debate rises from the fact that the establishment of hESC lines requires the destruction of a human embryo. For many, the early embryo represents a human being, and therefore the use of an embryo for research or medical purposes contradicts their moral and religious views. The problematic question is, at what stage of the development the embryo is regarded as a human being. Catholics regard embryos as human beings immediately after fertilizing, and thus the use of hESCs in research has been restricted in Catholic countries (Knowles 2010).

The regulations on hESC research vary greatly throughout the world, even inside the European Union. There are no universally uniform laws or ethical standards for the hESC research. In Finland, according to Medical Research Act 488/1999, the production of human embryos exclusively for research purposes is forbidden. Nevertheless, the derivation of hESC lines is allowed from embryos received from IVF treatments. It is forbidden to keep human embryos alive more than 14 days from their formation, excluding the time they have been kept frozen. (Ministry of Social Affairs and Health 2004.)

Stem cell study is an emerging research field, and it has become a largely discussed topic. Public discussion is often more based on emotions than on facts. All the aspects of hESC research need to be carefully considered, and clear rules and regulations should be stated. Stem cell study is developing fast so many countries are revising their present regulations in order to keep track of the dynamic field of the research. Stem cell research utilizing adult stem cells or iPSCs is considered less ethically problematic than the hESC research.

2.4. Differentiation of hESCs towards RPE cells

In the absence of appropriate intercellular signals designating cellular fate, embryonic stem cells choose neuronal differentiation (Tropepe et al. 2001; Smukler et al. 2006) and RPE cells are derivatives of this lineage. However, the induction of neural progenitor cells from hESCs should be maximized in order to get sufficient amounts of cells for research purposes. Especially for therapeutic applications it is crucial to

produce homogeneous cell populations. Generation of the RPE cells from the hESCs has several advantages because it can be done from pathogen-free cell lines under GMP conditions, and variations among batches can be kept minimal.

Numerous reports on producing RPE cells from the hESCs have been established. Differentiation can be induced in adherent culture (Klimanskaya et al. 2004; Klimanskaya 2006; Lund et al. 2006) or in suspension with EBs. Several differentiation protocols utilize both culture methods, at first the cells are cultured as EBs, and later passaged to adherent culture. To excite the RPE differentiation from the hESCs, mouse skull stromal PA6 feeder cells, MEF feeders, a culture medium lacking bFGF, or various supplements have been used. In addition, different coating materials have been utilized. PA6 is a stromal cell line derived from bone marrow. The differentiation time varies as it depends on the culture method and used cell lines, but for instance Klimanskaya (2006) have reported clusters of the hESC-RPE cells to appear usually in six to eight weeks. Idelson and co-workers have reported 51 % of hESC-RPE cell clusters to contain pigmented areas after four weeks of differentiation, and more than 70 % were pigmented after eight weeks. With a monolayer modification, 33 % of the cells were pigmented after six weeks of differentiation. (Idelson et al. 2009.)

Certain studies have demonstrated that PA6 co-culture can induce mouse and donkey embryonic stem cells into dopaminergic neurons and RPE cells (Kawasaki et al. 2000; 2002; Haruta et al. 2004). Gong and co-workers have used the mouse PA6 co-culture also in RPE differentiation from the hESCs. From MEF feeders, they seeded the hESCs onto the PA6 cells for 13 days. Finally, they passaged the cells onto coating materials for the RPE differentiation. Gong and co-workers' results showed that hESCs can be induced to differentiate into neural progenitors on PA6 co-culture, and the neural progenitors can be further differentiated into RPE cells (Gong et al. 2008.) However, Klimanskaya and co-workers have showed already in 2004 that such co-culture is not required for effective and reliable RPE differentiation (Klimanskaya et al. 2004; Klimanskaya 2006).

It has been suggested that for numerous hESC lines, the derivation of RPE cells may be spontaneous *in vitro* under MEF co-culture conditions. Prowse and co-workers have noticed that the culture conditions produced by the MEFs contain factors that may expose cells to the RPE differentiation. (reviewed by Vugler et al. 2007.) In fact, in numerous recent studies hESCs have been cultured on MEFs at the beginning of the RPE differentiation (Klimanskaya et al. 2004; Lund et al. 2006; Gong et al. 2008; Osakada et al. 2008; Vugler et al. 2008; Carr et al. 2009; Lu et al. 2009; Nistor et al. 2010). Nonetheless, the use of MEF feeders with human cells is not desirable since culture conditions for clinical applications should be xeno-free.

bFGF seems to repress RPE specification, and direct the differentiation of the embryonic optic vesicle towards neural retina (Martínez-Morales et al. 2004; Moshiri et al. 2004). Therefore several studies have used a culture medium lacking bFGF to excite the RPE differentiation from hESCs (Klimanskaya et al. 2004; Lund et al. 2006; Vugler et al. 2008; Carr et al. 2009). Idelson and co-workers have shown nicotinamide and

Activin A to promote the differentiation of hESCs towards RPE cells under defined culture conditions, but substituting Activin A with bFGF abolished the differentiation of hESCs towards RPE cells (Idelson et al. 2009). Nonetheless, Vugler and co-workers (2008) were unable to demonstrate any significant effect of the bFGF removal on pigment formation. On the other hand, Klimanskaya (2006) have reported that after the isolation of hESC-RPE cells, bFGF accelerates cell proliferation and reacquisition of the RPE morphology. Osakada and co-workers have successfully used Wnt and Nodal antagonists in feeder- and serum-free suspension culture to induce RPE cell differentiation from hESCs (Osakada et al. 2008). Wnt and Nodal signaling pathways are involved in the maintenance of hESC pluripotency (Sato et al. 2004; Vallier et al. 2005).

In addition to the neural retina, bFGF is suggested to affect also neural differentiation. Cohen and co-workers have suggested that hESCs differentiate into a primitive ectoderm-like fate independent of FGF-signaling, whereas further neural differentiation is instructed by FGF-signaling. This theory is in accordance with the ability of bFGF to promote the maintenance of undifferentiated hESCs. bFGF is used in several protocols for the neural induction of hESCs. (Cohen et al. 2010.) bFGF can also stimulate the transdifferentiation of RPE cells into neural retina (Galy et al. 2002) and neuronal progenitors (Opas & Dziak 1994).

Various coating materials have been used in hESCs differentiation towards RPE cells. Coating materials used to induce the differentiation or maintain hESC-RPE cell culture in recent studies are presented in Table 2.1.

Table 2.1. *Coating materials used to induce the differentiation of hESCs towards RPE cells or to maintain hESC-RPE cell culture.*

Coating material	Reference
Collagen I	Klimanskaya et al. 2004; Klimanskaya 2006
Collagen IV	Klimanskaya et al. 2004; Klimanskaya 2006
Collagen I/laminin	Nistor et al. 2010
Poly-D-lysine /laminin	Idelson et al. 2009
Poly-D-lysine/laminin/ fibronectin	Osakada et al. 2008
Laminin	Klimanskaya et al. 2004; Klimanskaya 2006; Idelson et al. 2009
Fibronectin	Klimanskaya et al. 2004; Klimanskaya 2006
Gelatin	Klimanskaya et al. 2004; Klimanskaya 2006; Lund et al. 2006; Carr 2009; Lu et al. 2009
Matrigel™	Gong et al. 2008; Vugler et al. 2008*; Carr et al. 2009

**Growth factor reduced Matrigel™*

In addition to these coating materials, Bruch's membrane has also been used to induce RPE differentiation from hESCs (Gong et al. 2008; Vugler et al. 2008). Some coating materials are discussed in more detail in Chapter 2.8.

So far, to my knowledge no study of hESC differentiation towards RPE cells with a xeno-free coating material and a serum-free culture medium in adherent cell culture has been published. In most of the preceding studies, differentiation was stimulated in EBs. In adherent cell culture studies, FBS is widely used to improve the cell attachment. Adsorption of FBS components on the cell culture surface alter its chemistry, and therefore influence subsequent cell attachment (Klauser et al. 2010).

2.5. Characterization of hESC-derived RPE cells

A common method of characterizing hESC-RPE cells is immunocytochemistry where a particular protein expression in the cell is detected. Typical early neural markers of RPE cells are transcription factors retina and anterior neural fold homeobox (RAX), paired box gene 6 (PAX6), and orthodenticle homeobox 2 (OTX2). RAX and PAX6 are homeobox genes expressed early in the development of an eye primordium. They regulate cell proliferation, and are essential for the initial determination to retinal and lens cell fate. (Furukawa et al. 1997; Mathers & Jamrich 2000.) The expression of PAX6 is required for the initial pigmentation of the RPE cells, but it is downregulated after early RPE differentiation, and absent in mature RPE cells (Martínez-Morales et al. 2004). OTX2 is also expressed in the early cells of the optic vesicle. OTX2-positive cell types have been identified as potential hESC-RPE precursors (Vugler et al. 2008), but later OTX2 is crucial for the cell fate specification of photoreceptors (Nishida et al. 2003). Vimentin and β-tubulin are also neural progenitor markers.

Typical molecular markers of mature RPE cells are cellular retinaldehyde-binding protein (CRALBP), retinal pigment epithelium-specific protein 65 kDa (RPE65), bestrophin, zonula occludens-1 (ZO-1), and microphthalmia-associated transcription factor (MITF). CRALBP binds to 11-*cis*-retinal or 11-*cis*-retinol in the visual cycle and is associated with normal dark adaptation (Saari et al. 2001). RPE65 is a cytoplasmic protein preferentially expressed in the RPE cells, and involved in the visual cycle and in vitamin A metabolism, therefore crucial for retinal function (Ma et al. 2001; Al-Hussaini et al. 2008). Bestrophin is a protein family that can function as a Cl^- channel as well as a regulator of voltage-gated Ca^{2+} channel (Hartzell et al. 2008). ZO-1 is a tight junction protein present in developing and mature RPE cells (reviewed by Vugler et al. 2008). MITF is essential for the acquisition of the pigmentation via tyrosinase activity and RPE cell identity maintenance. Mutations in the MITF gene cause lack of differentiated RPE cells and transdifferentiation of the RPE cells to non-pigmented neural retina (Bumsted & Barnstable 2000). Transcription factor ceh-10 homeodomain containing homolog (CHX10) is involved in the proliferation of non-pigmented retinal progenitors, so downregulation in the expression of MITF and other RPE-associated genes can be achieved by the misexpression of CHX10. Actually, the fate decision for the RPE cells versus the non-pigmented retina is mainly determined by the expression of MITF and CHX10, and the relationship between them. (Rowan et al. 2004; Vugler et al. 2007.) Sine oculis homeobox homolog 3 (SIX3) is a photoreceptor progenitor

(Vugler et al. 2007) whose downregulation can be assured in hESC-RPE cells. Many of the molecular markers used in immunocytochemical stainings can be used in RT-PCR, quantitative real time polymerase chain reaction (q-RT-PCR), and western blot. Immunocytochemistry and q-RT-PCR techniques are presented in the next chapter.

The phagocytic character of RPE cells can be assessed with phagocytosis assays. Pigmented cell clusters are incubated with fluorescent latex beads and stained, and finally the presence of latex beads in the cytoplasm of the RPE cells can be detected under a microscope (Klimanskaya 2006; Osakada et al. 2008; Idelson et al. 2009). Nevertheless, Carr and co-workers (2009) have suggested that the phagocytosis of latex beads is an inappropriate assay to measure hESC-RPE phagocytosis and the functional capacity of these cells, thus isolated POSs should be used.

2.6. Cell characterization techniques

2.6.1. Immunocytochemistry

Immunocytochemistry is a common laboratory technique that enables researchers to localize certain molecules from the cells, and visualize them under a microscope. Specific antibody binds to a specific protein or antigen in the cell. Antibodies, also known as immunoglobulins (Ig), are proteins shaped like Y letter and composed of four polypeptide chains. The binding of the antibody to the antigen can be visualized and examined under a fluorescence microscope, since antibodies can be labeled with fluorescent molecules.

Immunocytochemistry technique is composed of three steps. In the first step, the cells are fixed and permeabilized to ensure free access of the antibody to its antigen. Possible fixatives are paraformaldehyde, glutaraldehyde, methanol, or acetone, and they preserve the cells in a reproducible and life-like manner (Chemicon International 2010). They prevent the cells from shrinking, swelling, or dissolving cellular constituents, and they also protect the cells against the injurious effects of subsequent processing and staining procedures. In the second step, the cells are stained. Immunocytochemical staining can be direct or indirect. In the direct method, the used antibody is labeled with a detectable tag, for instance with a fluorescence molecule. The antigen can be visualized with the use of only one antibody. In the indirect method, also known as double staining, two antibodies are used. Primary antibody does not contain detectable tag, but it binds to the antigen. Labeled secondary antibody binds to the primary antibody, and therefore can be visualized. The secondary antibody must be generated against the immunoglobulins of the primary antibody source. For instance, if the primary antibody is raised in rabbit, the secondary antibody could be goat anti-rabbit. (IHC World 2010; Millipore 2010.) The indirect method is more sensitive, and the staining more intensive since every primary antibody is surrounded by several secondary antibodies (Törrönen 2006). Both primary and secondary antibodies should be diluted for each batch. Optimal antibody dilutions are the ones that give the strongest

specific antigen staining, but the lowest non-specific background. (Millipore 2010.) In the third step, the cells are examined under the fluorescence microscope. Usually the samples are compared to negative controls which establish background fluorescence and non-specific staining of the primary and secondary antibodies.

2.6.2. Quantitative real time PCR

Polymerase chain reaction (PCR) is a technique for an amplification of a desired fragment of deoxyribonucleic acid (DNA). In traditional PCR, the end product is the target of the analysis. The presence of a gene in question can be detected by gel electrophoresis, but the amount of the gene is unknown. Therefore, traditional PCR is called qualitative. q-RT-PCR is a technique to reveal the amounts of the genes.

q-RT-PCR is a widely used method of detecting and quantifying specific DNA sequences. This laboratory technique is based on the PCR which is used to amplify and simultaneously quantify a targeted DNA molecule. The amplified DNA is detected as the reaction progresses in real time, unlike in the traditional PCR. q-RT-PCR is based on the detection of a fluorescent reporter that increases as the PCR product accumulates with each amplification cycle. There are fluorescent reporters of two kinds: (1) non-specific dyes that bind any double-stranded DNA (i.e. SYBR Green I) and (2) sequence-specific DNA probes (i.e. TaqMan® Probes) (Sigma-Aldrich 2010).

In a q-RT-PCR protocol, at first total message RNA (mRNA) is extracted from the cells or tissues. The cells are lysed and mRNA purified. Complementary DNA (cDNA) is synthesized from mRNA molecules in a reaction catalyzed by enzyme reverse transcriptase. cDNA is a copy of mRNA, and therefore lacking the introns that are present in genomic DNA. In cDNA translation, the reverse transcriptase operates on a single strand of mRNA generating its cDNA based on the pairing rules. RNA bases A, U, G, and C pairs to their DNA complements T, A, C, and G respectively. A short oligonucleotide complementary to the poly-A tail at the 3´ end of the mRNA is first hybridized to the RNA to act as a primer for the reverse transcriptase. The reverse transcriptase along with deoxynucleoside triphosphates (A, T, G, and C) synthesizes one complementary strand of DNA which is hybridized to the original mRNA strand. RNA is enzymatically digested from the hybrid strand. Finally, the remaining single-stranded cDNA is copied into double-stranded cDNA. (Alberts et al. 2004.)

In the q-RT-PCR technique, a DNA-binding dye binds to the double-stranded DNA in PCR, which causes the fluorescence on the dye. An increase in DNA product during the PCR leads to an increase in fluorescence intensity which is measured at each cycle. TaqMan probes are largely used sequence-specific chemistries in q-RT-PCR. They depend on Förster Resonance Energy Transfer (FRET) to generate the fluorescence signal through the coupling of a fluorogenic dye molecule and a quencher moiety to the same or different oligonucleotide substrates. TaqMan probes are based on the 5´ -nuclease activity of DNA polymerase used for the PCR to hydrolyze an oligonucleotide which is hybridized to the target amplicon. These probes are oligonucleotides with a fluorescent reporter dye attached to the 5´ end and a quencher moiety attached to the 3´

end. During the PCR, when the polymerase replicates a template where a TaqMan probe is bound, the 5´ -nuclease activity of the polymerase cuts the probe. This separates the fluorescent and quenching dyes, and FRET no longer exists. Therefore, the fluorescence increases in each cycle, and it is commensurate to the amount of the probe cleavage. The fluorescence is detected and measured in a real time PCR thermocycler, and the increase in the fluorescence levels is used to determine the threshold cycle (C_T) in each reaction. (Dharmaraj 2010.) When analyzing the q-RT-PCR results, a comparative C_T method compares the C_T values of the samples with a control or calibrator. The C_T values of both samples and calibrators are normalized with an appropriate endogenous housekeeping gene. (Livak & Schmittgen 2001; Dharmaraj 2010.)

2.7. Cell-material interactions

Fundamental knowledge of cell-material interactions is crucial for tissue engineering and in the development of medical implants. Cell-material interactions may also explain differences in cell behavior in culture conditions. Yet, the cell-material interactions seem to be more largely studied on 3D-implants than on 2D-coating materials. Especially the implants for bone applications are widely examined. There are no universally optimal coating materials since the requirements for the coating material may vary depending on cell line and culture conditions. Essential elements when evaluating the cell-material interactions are adhesion proteins, material properties, and cell spreading.

2.7.1. Protein adsorption

In vitro, stem cells are usually cultured on rigid polystyrene tissue-culture plastics. Culture conditions on these plastics are very different from the conditions experienced by the cells in the body, where they are anchored to ECM proteins by discrete attachments in a relatively soft microenvironment. (Lutolf et al. 2009.) However, similarly than in the tissue, cell attachment to substrates or ECM components *in vitro* is normally mediated by adhesion proteins found in the serum-supplemented culture medium (Wilson et al. 2005; Yliperttula et al. 2008). Although the cell attachment and spreading may occur without serum proteins, culture surfaces are nonphysiological surfaces which can decrease cell activity and contribute to cell death (Wilson et al. 2005). Culture medium proteins, for instance fibronectin, laminin, and collagen, interact with specific receptors on the cell surface (Yliperttula et al. 2008).

The primary interaction between the cells and the adhesion proteins occurs via integrins which function as cell surface receptors. (Wilson et al. 2005.) Integrins are members of the family of cellular adhesion molecules that are expressed by several cell types (Gräber et al. 1999). This primary interaction has been demonstrated by introducing antibodies to prevent these interactions, which has lead to the decrease in cell attachment. Even though this initial interaction is critical, the system between the cells and the culture surface is dynamic. Cells may alter their attachment mechanism

and environment by secreting fibronectin or by manipulating the ECM. In addition, cells have the ability to adapt to their environment, and therefore protein production may alter depending on the culture conditions. (Wilson et al. 2005.) For instance, it has been discovered that collagen production of osteoblasts increases on rough surfaces (Martin et al. 1995).

Proteins have a significant surface activity, and therefore exhibit high affinity for interfaces. Protein adsorption to the culture surfaces is also fast. Initial adsorption occurs rapidly, and it effectively prevents direct interactions between the cells and the culture surfaces. (Wilson et al. 2005.) Protein adsorption is a complex process, and it may be promoted or opposed by potential enthalpic and entropic changes. These changes include partial dehydration of proteins and sorbent surfaces, the redistribution of charged groups in the interface, and conformational changes in the protein molecule. (Haynes & Norde 1994.) The adsorption rate usually decreases in relation to the available binding sites, and it becomes gradually more dependent on the protein-surface affinity. On the whole, the relative concentrations of the proteins and their surface affinities determine which proteins adsorb to a limited number of binding sites. (Wilson et al. 2005.)

For a protein to adsorb to the culture surface, both adsorbate and the surface must at least partially dehydrate. This is thermodynamically favorable for hydrophobic sorbents and adsorbates. Meanwhile, the displacement of water molecules from hydrophilic surfaces presents a considerable energy barrier to protein adsorption. Hydrophobic surfaces are normally reported to adsorb more protein than hydrophilic surfaces, probably due to greater number of possible adsorption-promoting interactions. However, some proteins display a high charge-dependent surface affinity. (Wilson et al. 2005.)

Opposite charged proteins and culture surfaces are attracted. Nevertheless, the situation becomes more complicated in aqueous solutions, where surface charges are shielded by hydrating water, modulated by pH, and counterbalanced by small ions. Interactions may occur at the atomic scale, but the global charges of the proteins and the surfaces dominate electrostatically driven adsorption. (Haynes & Norde 1994.)

In addition to the dehydration effects, the conformational changes of the protein affect the entropy of the system, and therefore the protein adsorption. The conformational change is the main driving force for the protein adsorption to hydrophilic surfaces or surfaces carrying a like charge to the protein. Studies have shown that some conformational changes occur in most protein adsorption, but the adsorbed proteins are not completely denatured on adsorption. The activity of an adsorbed protein depends on its conformation and orientation. (Wilson et al. 2005.)

2.7.2. Material characteristics

Material properties affect the cell attachment. The cell adhesion and the following activity are mostly superior on hydrophilic surfaces than on hydrophobic surfaces. Several resorbable polymers used in tissue engineering are hydrophobic, and require

surface modification or wetting before the cell passage. One reason for the different cell response on hydrophilic and hydrophobic materials is the difference in the array of proteins adsorbed to the surfaces. For instance, vitronectin is usually more adsorbed to hydrophilic than hydrophobic surfaces. (Wilson et al. 2005.)

The process of the cell adhesion is different for positive and negative charges. Cell membranes attach closely to positively charged surfaces, whereas there are only distinct contact points on nearly neutral and negatively charged surfaces. (Wilson et al. 2005.) This has been discovered in osteoblast studies (Davies et al. 1986; Shelton et al. 1988). Electrostatic attraction between the cell membrane and the culture surface enhances the cell attachment, but it does not necessary support subsequent functions, including cell spreading and differentiation. The ionic content of the culture surface is probably significant in determining the composition of the adsorbed proteins by minimizing similar charges. (Wilson et al. 2005.) Some cells have difficulties in attaching negatively charged surfaces. Most commonly used substrata in the cell culture are plates with plastic wells which offer negatively charged surfaces. Some anchorage-dependent cells adhere only weakly to the plastic surface if they do not produce sufficiently positively charged ECM proteins, or are unable to utilize proteins in the culture medium. In this case, coating the plastic well surface usually facilitates the cell attachment. (reviewed by Vancha et al. 2004.)

Surface roughness and topography also influence the cell attachment. Generally, the cell attachment is increased on rough surfaces. The roughness and disorders of the surface increase the surface area. They also create confined spaces. It has been proposed that the confined spaces may interfere with the wetting of hydrophobic surfaces, lead to a localized dilution of the coating material solution, or restrict the exchange between the surface and the solution. It has also been suggested that topographic effects on protein adsorption relate to an increase in surface energy with roughness. Interaction with the material may affect cell properties. For instance, cell morphology, motility, proliferation, and differentiation may change. Normally the cell motility and attachment show opposite trends: if the cell is tightly attached to the surface, cell motility is minor. (Wilson et al. 2005.) Changes in cell morphology can be studied by using microscopic techniques like phase contrast and electron microscopy (Kumari et al. 2002). However, all of these reactions depend on cell type and material.

2.7.3. Cell spreading

Contact sites in the cell attachment can be classified as focal contacts, close contacts, and extracellular contacts, and the contact type depends on the distance of the cell from the substratum and the presence of certain proteins (Kumari et al. 2002). Focal contacts are the contacts where the plasma membrane and substratum leave a gap of only ~10-15 nm (Zamir & Geiger 2001; Lemire et al. 2002). Focal contacts are associated with actin filaments at their cytoplasmic aspects, and seem to be significant in the regulation of actin organization, thereby affecting cell spreading, migration, and morphogenesis

(Zamir & Geiger 2001). Close contacts are the contacts where the distance between the plasma membrane and substratum is ~30 nm (Lemire et al. 2002).

The cell spreading is a process related to the attachment, and it often involves the same ECM proteins. These proteins are required for the formation of the main contacts and essential intracellular structures. As the cells spread, contractile forces apply tension to the ECM, and these forces may be sufficient to remove the adsorbed proteins from the material surface. *In vitro,* several cell types have been shown to depend especially on adsorbed vitronectin and fibronectin for initial spreading, for instance on tissue culture polystyrene. Therefore, the ability of materials to adsorb those proteins from serum is crucial in supporting the cell adhesion and spreading. (Wilson et al. 2005.) Serum-free culture conditions are more challenging for cell attachment.

2.8. Coating materials

This chapter introduces diverse coating materials used in the laboratory experiments of this thesis. Materials are briefly presented and some examples of the studies using these materials are described. In most cases only one coating material has been studied, but the mixtures of several coating materials can also be effective to promote the cell attachment and differentiation.

2.8.1. CELLstart™

CELLstart™ (Gibco, Invitrogen, Carlsbad, CA, USA) is a commercial xeno-free coating material for cell culture. Its composition is unknown, but most probably it contains different ECM proteins and no growth factors. According to the manufacturer, CELLstart™ supports the hESC attachment and expansion of undifferentiated colonies in a serum-free medium. Feeder cells are unnecessary when using CELLstart™ coating. (Gibco, Invitrogen 2008.) CELLstart™ has been used in hESC research (Ojala 2009), but also in adipose stem cell culture (Lindroos et al. 2009; Rajala et al. 2010).

2.8.2. Collagen-based coating materials

Collagen is the most abundant protein in the human body, and it is the major component of the skin and other musculoskeletal tissues. Collagen superfamily consists of 28 different types (Veit et al. 2006), with the most common being types I-IV. The primary structure of the collagens consists of repeating triplets of Glysine-X-Y, where X and Y are usually proline and hydroxyproline. (Nair & Laurencin 2007.) Collagen molecules have a tendency to aggregate into fibrils, and the arrangement of the fibrils to a higher organization differs between collagen types. In addition, different tissues have diverse collagen compositions. (Kühn 1985.) In the body, collagen serves as a natural substrate for cell attachment and degrades enzymatically. Highly reactive collagen dissolves easily in acidic aqueous solutions, and can be processed into various forms. The degradation rate of collagen used for biomedical applications can be altered by

enzymatic pre-treatment or cross-linking using cross-linking agents. (Nair & Laurencin 2007).

In 1986, Campochiaro and co-workers showed that the ECM of human RPE cells contains collagen types I-IV (Campochiaro et al. 1986). Types I, II, and III constitute the family of interstitial collagens, and they are homologous. Their molecules are rod-shaped with a molecular weight of approximately 300 000 g/mol, and the length of about 300 nm. (Kühn 1985.)

Collagen is one of the most used supporting matrix for the growth and maturation of hESC-derived neuronal cells (Auer et al. 2009). Gelatin, which is the denatured form of collagen, is also a widely used coating material. It is typically isolated from bovine or porcine skin or bone by acid or base extraction (Olsen et al. 2003).

2.8.2.1 VitroCol™

VitroCol™ (Advanced BioMatrix, Inc., San Diego, CA, USA) is a commercial human collagen solution for cell culture. According to the manufacturer, VitroCol™'s composition is about 97 % collagen I with the remaining part being collagen III, and it is prepared from the extracellular matrix secreted by normal human fibroblasts (Advanced BioMatrix, Inc. 2009).

Collagen I, the main component in VitroCol™, has been studied as a coating material for RPE cells. Klimanskaya and co-workers (2004) have succeeded in obtaining consistent differentiation of hESCs towards RPE cells on collagen I coating.

2.8.2.2 Collagen IV

Unlike most collagens, collagen IV is found only in the basement membranes, and it consists of six genetically distinct α-chains. Through complex inter- and intramolecular interactions, collagen IV forms supramolecular networks that influence cell migration, attachment, and differentiation. (Khoshnoodi et al. 2008.) Its molecules are 400 nm in length with a molecular weight of approximately 600 000 g/mol (Kühn 1985).

In addition to collagen I, Klimanskaya and co-workers (2004) have succeeded in obtaining consistent differentiation of hESCs towards RPE cells on collagen IV coating. Ho and Del Priore (1997) have studied the attachment of human harvested RPE cells to RPE-derived ECM and Bruch's membrane. They discovered that the RPE attachment rate was higher in collagen IV coated ECM and Bruch's membrane than in uncoated ECM and Bruch's membrane. (Ho & Del Priore 1997.)

There are also some negative results of collagen IV as a coating material. Braam and co-workers (2008) have studied synthetic ECM substrates as alternative coating materials to Matrigel™. In their experiments, hESCs attached poorly to collagen IV when using defined medium supplements. (Braam et al. 2008.)

2.8.3. Poly-L-lysine

Poly-L-lysine is a small polypeptide composed of essential amino acid L-lysine. As being a synthetic cation, it has been used in several studies as an attachment promoting factor. For instance, poly-L-lysine or poly-D-lysine has often been used for pre-coating plates for PC-12 (derived from rat pheochromocytoma) cell culture (Turner et al. 1989; Zhuo et al. 2003; Vancha et al. 2004).

Yavin and Yavin (1974) have studied the attachment and the culture of dissociated cells from rat embryo cerebral hemisphere in poly-L-lysine coated plastic Petri dishes. Untreated plastic Petri dishes were used as controls. Almost 90 % of the cells were attached to poly-L-lysine treated surfaces after 20 minutes incubation. On the contrary, most of the cells did not attach to untreated Petri dishes. In addition, the detachment from poly-L-lysine treated surfaces was minor, unlike from untreated surfaces. The attachment of the cells on poly-L-lysine coated surfaces was followed by cell proliferation and characteristics of neural tissue differentiation. (Yavin & Yavin 1974.)

2.8.4. Polyethyleneimine

Polyethyleneimine (PEI) is a synthetic, organic macromolecule with a high cationic-charge-density potential. Every third atom in the backbone is nitrogen that can be protonated. (Boussif et al. 1995.) Due to its cationic character, PEI is widely investigated as a gene carrier. Cationic polymers can spontaneously condense DNA, which is a necessity for gene transfer in most cell types. (De Smedt et al. 2000.)

Vancha and co-workers (2004) have studied PEI as an attachment factor with two commonly used cell lines, PC-12 and HEK-293 (human embryonic kidney). Both tested cell lines attached firmly to tissue culture dishes coated with PEI. Vancha and co-workers also noticed that PEI enhanced the attachment of zebrafish retinal explants to the tissue culture dishes. Therefore PEI seems to be an efficient attachment factor for primary cells and weakly anchoring cell lines. (Vancha et al. 2004.) Earlier PEI was used as a coating material to promote the attachment of neurons in primary cell cultures (Rüegg & Hefti 1984; Lelong et al. 1992).

2.8.5. Fibronectin

Fibronectin is a high molecular weight glycoprotein in the ECM. It exists as a dimer composed of two almost identical ~250 kDa subunits that are covalently linked together by a pair of disulfide bonds. Fibronectin participates in a wide range of cellular interactions, and plays a significant role in cell attachment, differentiation, growth, and migration. (Pankov & Yamada 2002.)

The attachment of RPE cells to fibronectin coating has been studied. In addition to collagen I and IV, Klimanskaya and co-workers (2004) have succeeded in obtaining consistent differentiation of hESCs towards RPE cells on fibronectin coating. Tezel and Del Priore (1996) have studied the attachment of human RPE cells to fibronectin coated

plastic tissue culture dishes. They discovered that fibronectin coated dishes supported the human RPE cell attachment better than uncoated plastic tissue culture dishes. However, the cell attachment was better to ECM coated dishes than to fibronectin coated dishes. Likewise the cell attachment studies, Tezel and Del Priore have studied apoptosis rates on different coating materials, and the results were parallel to the cell attachment. The rate of the apoptosis in the human RPE cell culture was the lowest in the ECM coated dishes, followed by fibronectin coated dishes and uncoated plastic tissue culture dishes. Nonetheless, the differences in the apoptosis rates in the ECM or fibronectin coated dishes were not significant. (Tezel & Del Priore 1996.)

Ho and Del Priore (1997) have had similar results in their research. They discovered that RPE-derived ECM and Bruch's membrane coated with fibronectin had an increased human RPE cell attachment rate in comparison with uncoated ECM and Bruch's membrane. (Ho & Del Priore 1997.) Fibronectin is also one of the most used supporting matrix for the growth and maturation of hESC-derived neuronal cells (Auer et al. 2009).

2.8.6. Vitronectin

Vitronectin is an adhesive glycoprotein found in the plasma and in the ECM of tissues (Hayman et al. 1983). It plays a crucial role in attaching the cells to their surrounding matrix, and may be involved in the regulation of cell proliferation, differentiation, and morphogenesis. Vitronectin participates also in haemostasis and vascular remodeling by for instance interacting with thrombin and antithrombin III during coagulation. Liver seems to be the major source of plasma vitronectin. (Preissner & Seiffert 1998.)

Human vitronectin has a molecular mass of 75 kD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without reduction. Under reducing conditions, it circulates as single-chain (75 kD) and two-chain (10 kD and 65 kD) forms. Two fragments in the two-chain form are held together by a disulfide bridge. (Preissner & Seiffert 1998.)

Ho and Del Priore (1997) have studied the effect of vitronectin on RPE cell attachment. Likewise collagen IV and fibronectin, RPE-derived ECM and Bruch's membrane coated with vitronectin had an increased human RPE cell attachment rate in comparison with uncoated ECM and Bruch's membrane. (Ho & Del Priore 1997.)

In Braam and co-workers' (2008) experiments, the hESCs attached efficiently to natural and recombinant vitronectin. In addition, recombinant vitronectin supported sustained self-renewal and pluripotency in tested hESCs lines, and seemed to be a functional alternative to Matrigel™. (Braam et al. 2008.)

2.8.7. Laminin

Laminins are a family of heterotrimeric glycoproteins in the ECM. They exist as a cruciform-like structure formed by three disulphide-bonded chains: α, β, and γ. Laminins are the first ECM molecules synthesized in the developing embryo. The main role of the laminins is in cell-matrix attachment, but multiple additional biological

activities have been recognized. For instance, promoting cell growth, migration, and wound repair have been demonstrated. (Malinda & Kleinman 1996.)

In addition to collagen I and IV, and fibronectin, Klimanskaya and co-workers (2004) have succeeded in obtaining consistent differentiation of hESCs towards RPE cells on laminin coating. Tezel and Del Priore (1996) found out in their experiments that laminin coated plastic tissue culture dishes supported the human RPE cell attachment better than the uncoated plastic tissue culture dishes. However, the cell attachment was better to ECM coated dishes than to laminin coated dishes. In addition, the cell attachment was slightly better to fibronectin coated dishes than to laminin coated dishes. The apoptosis rates on different coating materials were parallel to the cell attachment. Laminin coated dishes had lower apoptosis rates than the uncoated plastic tissue culture dishes, but higher apoptosis rates than the ECM or fibronectin coated dishes. Nevertheless, the differences in the apoptosis rates in the ECM, fibronectin, or laminin coated dishes were not significant. (Tezel & Del Priore 1996.)

Ho and Del Priore (1997) discovered in their studies that laminin coating supported human RPE cell attachment. RPE-derived ECM and Bruch's membrane coated with laminin had an increased RPE cell attachment rate compared to uncoated ECM and Bruch's membrane. (Ho & Del Priore 1997.) Laminin is also one of the most used supporting matrix for the growth and maturation of hESC-derived neuronal cells (Auer et al. 2009).

2.8.8. Nidogen

Nidogen family, also called entactins, is composed of multiple sulfated monomeric glycoproteins present in the basement membrane. Nidogen-1 (150 kD) have a modular structure with three globular domains: G1-G3. (Ho et al. 2008.)

Nidogens have a wide repertoire of binding partners, especially the other basement membrane components collagen IV, laminin, perlecan, and fibulin. G1 is reported to bind collagen IV, G2 binds perlecan, and G3 binds laminin. Due to these interactions, nidogens are presumed to connect and stabilize the major networks of the basement membrane, and to target mesenchymal nidogen to the epithelial or endothelial basement membranes. (Ho et al. 2008.)

Although nidogens are omnipresent components of the ECM, and have multiple binding partners, a genetic analysis has proved that they are not necessary for the overall architecture of the basement membrane. Their significant role is to stabilize the tissues undergoing rapid growth or turnover. (Ho et al. 2008.)

In Braam and co-workers' (2008) experiments hESCs attached poorly to nidogen+laminin when using defined medium supplements. Therefore, at least the combination of nidogen and laminin does not seem to be as effective as Matrigel™ in hESC culture. (Braam et al. 2008.)

3. RESEARCH METHODS AND MATERIALS

3.1. Cell material

Three hESC lines were used in this study: Regea 06/040, Regea 08/013, and Regea 08/023. Cell lines have been derived from early stage embryos donated for research by couples undergoing IVF treatments. Regea has the approval of the Ethics Committee of Pirkanmaa Hospital District to derive and culture the three hESC lines used in this study. All the cell lines are also regularly characterized in Regea. Regea 06/040 cell line has karyotype 46, XX, and Regea 08/013 and Regea 08/023 have karyotype 46, XY. All cell material used in this study was cultured in humified HeraCell 150 and 240 incubators (Thermo Electron Corporation, Thermo Fisher Scientific Inc., Walthan, MA, USA) at +37 ºC in 5 % carbon dioxide atmosphere.

hESC lines were cultured on commercially available human foreskin fibroblast (hFFs) (CRL-2429, American Type Culture Collection ATCC, Manassas, VA, USA) feeder cell layer which had been mitotically inactivated with gamma irradiation (40 Gy). hESCs were cultured on the feeder cells until transferring to feeder-free culturing systems at the beginning of the experiments. The hESCs were cultured in HES medium containing Knockout™ D-MEM (Gibco, Invitrogen) supplemented with 20 % Knockout™ Serum Replacement (Gibco, Invitrogen), 2 mM GlutaMAX™-l Supplement (Gibco, Invitrogen), 1 % MEM Non Essential Amino Acids (Camprex BioScience East Rutherford, NJ, USA), 0.5 % Penicillin/Streptomycin (Cambrex), 0.1 mM 2-Mercaptoethanol (Gibco, Invitrogen), and 8 ng/ml human bFGF (R&D Systems, Minneapolis, MN, USA). Cells were monitored and the culture medium was changed 6 times per week. Five to eight days after plating, the undifferentiated areas of the hESC colonies were passaged onto fresh hFF feeder cells by mechanical disaggregation.

RPE cell differentiation was induced in feeder-free culture with RPE DM- medium which contained Knockout™ D-MEM (Gibco, Invitrogen) supplemented with 15 % Knockout™ Serum Replacement (Gibco, Invitrogen), 2 mM GlutaMAX™ –l Supplement (Gibco, Invitrogen), 1 % MEM Non Essential Amino Acids (Camprex), 0.5 % Penicillin/Streptomycin (Camprex), and 0.1 mM 2-mercaptoethanol (Gibco, Invitrogen). The composition of RPE DM- medium was almost the same as the composition of HES medium, but it did not contain bFGF. Fresh RPE DM- medium was prepared weekly.

Cell culture experiments are illustrated in Figure 3.1. The horizontal arrow represents the differentiation of hESCs towards RPE cells. The experiments are presented below the arrow at the time point of the differentiation when the experiment begins. Above the arrow are presented the typical markers of hESCs, neural progenitors,

and RPE cells. Some of these typical markers of the neural progenitors and the RPE cells are used in cell characterization experiments.

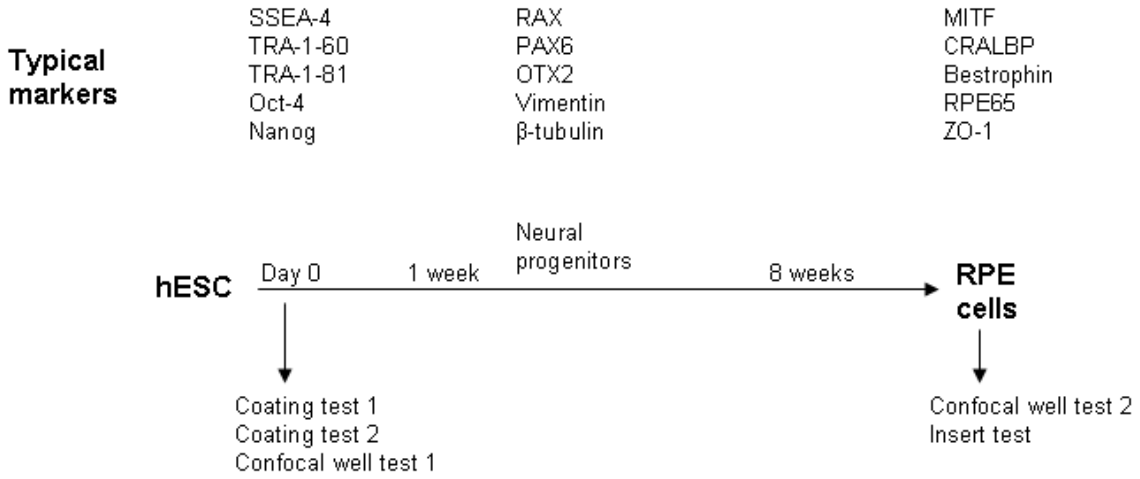

Figure 3.1. *Schema of the experiments on different time points during the RPE differentiation from hESCs.*

3.2. Cell culture on different coating materials

Cell culture on different coating materials consists of Coating test 1 and Coating test 2. Coating protocols and hESC passage of these experiments are described in this chapter.

3.2.1. Coating test 1: coating protocols

The attachment of hESCs, which were differentiating towards RPE cells, to the well surface was tested with five different basic materials, and with some additional proteins and different combinations. Basic coating materials were CELLstart™ (Gibco, Invitrogen), collagen IV (Sigma-Aldrich, St. Louis, MO, USA), VitroCol™ (Advanced BioMatrix), poly-L-lysine (Sigma-Aldrich), and PEI (Sigma-Aldrich). Additional proteins were fibronectin, vitronectin, and laminin, all from Sigma-Aldrich. Tested coating materials were chosen based on previous publications and research group's former results. All tested materials were xeno-free. The volume of the coating solutions added to a well in 24-well plate (Nunclon™, Sigma-Aldrich) was 230 µl. Coating protocols are presented in Table 3.1. Biological replicates stand for parallel samples.

CELLstart™ was diluted 1:50 in Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza Group Ltd, Biowhittaker, Basel, Switzerland). Diluted CELLstart™ was added to the wells in a volume of 120 µl/cm^2, which is bigger than the manufacturer's instructions. The amount of the coating material that the manufacturer recommends did not seem to cover the well properly. 120 µl/cm^2 of diluted CELLstart™ corresponds to 2.4 µl/cm^2 of undiluted CELLstart™. Coating was incubated (Table 3.1), after which the unattached coating material was removed. Finally RPE DM- medium was added to the wells for the coming hESC passage.

Additional proteins were coated with CELLstart™. Two different protocols were used with the protein coating: (1) mixing the protein with the basic coating material and (2) coating first the basic coating material and then the protein on the basic material. In the first protocol, the additional protein and the basic coating material were mixed together, added to the well, and incubated at the same time. In the second protocol, the additional protein was diluted in DPBS to the final volume (230 µl). After that the protein and DPBS solution was added to the CELLstart™ coated wells and incubated. In Table 3.1, the incubation information 2h, + 37ºC – 1h, RT for CELLstart™+ fibronectin (in DPBS) means that at first CELLstart™ in DPBS was coated for two hours at + 37 ºC after which fibronectin in DPBS was coated on CELLstart™ coating for one hour at room temperature (RT). In both protocols, the wells were washed twice with DPBS, and RPE DM- medium was added like before. Washing and RPE DM- medium addition were performed to all coatings described below.

Collagen IV was first diluted in acetic acid (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. After that, collagen IV was diluted in DPBS to the final volume and coated to the wells. Collagen IV and CELLstart™ were also coated together with DPBS. Third collagen IV coating contained fibronectin, vitronectin, and Nidogen-1/Entactin (R&D Systems) which were all mixed together with DPBS using the first protein coating protocol.

VitroCol™ is quite acidic (pH 2), so it had to be neutralized before coating. 8 parts of chilled VitroCol™ solution were mixed with 1 part of chilled 10 x DPBS (Lonza Group Ltd, Biowhittaker). pH of this mixture was adjusted to about 7.5 with sterile 0.1 M sodium hydroxide (Sigma-Aldrich) in ice. VitroCol™ became unsterile in this neutralization procedure, which has to be taken into account when analyzing the results.

Undiluted poly-L-lysine was coated with the additional proteins using only the first protein coating protocol. The proteins were diluted in poly-L-lysine to the final volume.

PEI was diluted 1:1000 in sterile water. Diluted PEI was coated with additional proteins, and both protein coating protocols were used. In the first protocol, the proteins were diluted in PEI to the final volume. In the second protocol, the proteins were diluted in DPBS.

Table 3.1. *Coating protocols in Coating test 1. Used cell line was Regea 08/013. Material/material stands for the first protein coating protocol, and material+material stands for the second protein coating protocol.*

Coating material	Coating concentration	Incubation	Biological replicates
CELLstart™ (in DPBS)	2.4 µl/cm²	2h, +37ºC	3
CELLstart™/fibronectin (in DPBS)	2.4 µl/cm² / 5 µg/cm²	2h, +37ºC	1
CELLstart™+fibronectin (in DPBS)	2.4 µl/cm² + 5 µg/cm²	2h, +37ºC – 1h, RT	2
CELLstart™/vitronectin (in DPBS)	2.4 µl/cm² / 1 µg/cm²	2h, +37ºC	1
CELLstart™+vitronectin (in DPBS)	2.4 µl/cm² + 1 µg/cm²	2h, +37ºC – 1h, RT	2
CELLstart™/laminin (in DPBS)	2.4 µl/cm² / 5 µg/cm²	2h, +37ºC	1
CELLstart™+laminin (in DPBS)	2.4 µl/cm² + 5 µg/cm²	2h, +37ºC – 1h, RT	2
Collagen IV (in DPBS)	5 µg/cm²	2h, +37ºC	2
Collagen IV/CELLstart™ (in DPBS)	5 µg/cm² / 2.4 µl/cm²	2h, +37ºC	2
Collagen IV/fibron./vitron./nidogen (in DPBS)	5 µg/cm² / 5 µg/cm² / 1 µg/cm² / 1 µg/cm²	2h, +37ºC	2
VitroCol™	120 ul/ cm²	2h, + 37 ºC	2
Poly-L-Lysine/fibronectin	120 µl/cm² / 5 µg/cm²	2h, +37ºC	2
Poly-L-Lysine/vitronectin	120 µl/cm² / 1 µg/cm²	2h, +37ºC	2
Poly-L-Lysine/laminin	120 µl/cm² / 5 µg/cm²	2h, +37ºC	2
PEI/fibronectin	120 µl/cm² / 5 µg/cm²	o/n, RT	1
PEI+fibronectin	120 µl/cm² + 5 µg/cm²	2h, RT – o/n, RT	2
PEI/vitronectin	120 µl/cm² / 1 µg/cm²	o/n, RT	1
PEI+vitronectin	120 µl/cm² + 1 µg/cm²	2h, RT – o/n, RT	2
PEI/laminin	120 µl/cm² / 5 µg/cm²	o/n, RT	1
PEI+laminin	120 µl/cm² + 5 µg/cm²	2h, RT – o/n, RT	2

Abbreviations: fibron: fibronectin; vitron: vitronectin; RT: room temperature; o/n: overnight.

3.2.2. Coating test 1: hESC passage to the coated wells

From the cell culture on hFFs in HES medium, hESCs were passaged mechanically to the coated wells. Used cell line in Coating test 1 was Regea 08/013. RPE DM- medium had already been added to the wells. Undifferentiated areas of hESC colonies were cut mechanically with a scalpel, and the cells were passaged into the wells with a needle and a pipette. 20-35 small colony pieces were added to each well. The cells were

cultured at +37 ºC, and RPE DM- medium was changed three times per week. Cell attachment was monitored under a stereomicroscope (Nikon, SMZ800, Nikon Instruments Europe B.V. Amstelveen, The Netherlands) and a phase contrast microscope (Eclipse TE2000-S, Nikon Instruments) daily.

The cell attachment and growth were classified as good, very good, or low based on visual observations. Good hESC cluster attachment on day two means that several hESC clusters had attached to the wells. Very good hESC cluster attachment on day two means that the attached hESC clusters had started to form colonies. Low hESC cluster attachment on day two means that only few hESC clusters had attached to the wells. Good cell growth in later time points (day seven and day 14) means that the colonies had grown, and few cells were detaching. Very good cell growth in later time points (day seven and day 14) means that the colonies had grown well, and very few cells were detaching. Low cell growth in later time points (day seven and day 14) means that the colonies had not grown, and several cells were detaching. However, the most important criterion in the classification is the comparison of the cells and the colonies on different coating materials. Cell culture was carried on for 15 days.

3.2.3. Coating test 2

Based on the results from Coating test 1, further studies in Coating test 2 were performed with CELLstart™, collagen IV, and VitroCol™. The coating protocols with all three materials and hESC passages were carried out as before. However, two cell lines were used for all materials: Regea 06/040 and Regea 08/013. In addition, two well plate formats were used: 24-well plate and 48-well plate (Nunclon™, Sigma-Aldrich) (Table 3.2). Concentrations of the coating materials were the same in 48-wells than in 24-wells, but the volume of the coating solution added to a well in a 48-well plate was 135 µl. The volume of the coating solution added to a well in a 24-well plate was 230 µl as before. RPE DM- medium was changed three times per week, and cell attachment and pigment formation were monitored under a stereomicroscope (Nikon, SMZ800, Nikon Instruments) and a phase contrast microscope (Eclipse TE2000-S, Nikon Instruments) daily. The cell attachment and growth were classified as good, very good, or low based on visual observations, and the classification criteria were the same as in Coating test 1. In pigment forming observation, brown areas on the colonies were searched visually under a stereomicroscope. The cells in 24-well plates were used for RNA isolations in three different time points, and the cells in 48-well plates were fixed on day 21 for immunocytochemical stainings.

Table 3.2. *Coating protocols in Coating test 2.*

Coating material	Coating concentration	Incubation	Cell line (Regea)	Well plate format	Biological replicates
CELLstart™	2.4 µl/cm²	2h, +37ºC	06/040	24	6
CELLstart™	2.4 µl/cm²	2h, +37ºC	08/013	24	6
CELLstart™	2.4 µl/cm²	2h, +37ºC	06/040	48	2
CELLstart™	2.4 µl/cm²	2h, +37ºC	08/013	48	2
Collagen IV	5 µg/cm²	2h, +37ºC	06/040	24	6
Collagen IV	5 µg/cm²	2h, +37ºC	08/013	24	6
Collagen IV	5 µg/cm²	2h, +37ºC	06/040	48	2
Collagen IV	5 µg/cm²	2h, +37ºC	08/013	48	2
VitroCol™	120 ul/cm2	2h, +37ºC	06/040	24	6
VitroCol™	120 ul/cm2	2h, +37ºC	08/013	24	6
VitroCol™	120 ul/cm2	2h, +37ºC	06/040	48	2
VitroCol™	120 ul/cm2	2h, +37ºC	08/013	48	2

3.3. Confocal microscopy wells

Cell attachment to confocal microscopy wells was tested in this experiment. In confocal well test 1, hESCs were passaged to commercially coated Ibidi wells. In confocal well test 2, hESC-RPE cells were passaged to self-coated Ibidi wells.

3.3.1. Confocal well test 1

Three different commercially coated confocal microscopy wells were tested: collagen IV, ibiTreat, and poly-L-lysine. All these plates were from Ibidi GmbH, München, Germany and had 8 wells. The composition of IbiTreat coating was unknown. Two cell lines were used in Confocal well test 1: Regea 06/040 and Regea 08/013. hESCs were passaged to the wells as before, 15-25 small colony pieces per well. In each 8-well coated plates, there were four biological replicates of both cell lines. Cells were cultured at +37 ºC, and RPE DM- medium was changed three times per week. Pigment formation and cell attachment were monitored under a stereomicroscope (Nikon, SMZ800, Nikon Instruments) and a phase contrast microscope (Eclipse TE2000-S, Nikon Instruments) daily. The cell attachment and growth were classified as good, very good, low, or very low based on visual observations. Very low cell attachment and growth means that there were very few attached cells and small colonies in the wells, otherwise the classification criteria were the same as in Coating test 1. Also in this case, the most important criterion in the classification is the comparison of the cells and the

colonies on different coating materials. Cell culture was carried on for 21 days, until the cells were fixed for immunocytochemical stainings. Some of the wells were emptied earlier by disgorging the unattached cells.

3.3.2. Confocal well test 2

Uncoated confocal microscopy wells (Ibidi GmbH) were coated with CELLstart™ and collagen IV according to the protocols described previously. In this experiment, mature RPE cells differentiated from hESCs were used. Pigmented cell clusters in suspension were degraded by using two different protocols: mechanically with a scalpel and a needle or enzymatically. In the mechanical dissection, hESC-RPE cells differentiated from cell line Regea 08/023 were used. 20-30 cut small pigmented cell clusters were passaged to each well. Cells were cultured at +37 ºC, and RPE DM- medium was changed twice per week. Cell attachment and growth were monitored under a stereomicroscope (Nikon, SMZ800, Nikon Instruments) and a phase contrast microscope (Eclipse TE2000-S, Nikon Instruments) for three weeks. RPE cell cluster attachment and growth were classified as good, very good, low, or very low based on visual observations. Good RPE cell cluster attachment means that several hESC-RPE cell clusters had attached to the wells. Very good RPE cell cluster attachment means that the attached hESC-RPE cell clusters had grown to the surroundings. Low RPE cell cluster attachment means that few hESC-RPE cell clusters had attached to the wells. Very low RPE cell cluster attachment means that very few hESC-RPE cell clusters had attached to the wells. Also in this case, the most important criterion in the classification is the comparison of the cells and the colonies on different coating materials. Coating protocols in Confocal well test 2 are presented in Table 3.3.

Enzymatic cell cluster degradation was performed by using trypsin (Lonza Group Ltd). 10 x trypsin was diluted 1:10 in DPBS. hESC-RPE cells differentiated from cell line Regea 08/013 were used in this experiment. At first, pigmented cell clusters were washed twice with DPBS which was finally removed. After that, diluted trypsin was added to the wells (260 µl/cm^2). Cell clusters with trypsin were incubated at +37 ºC for an hour, after which the cell clusters were pipetted back and forth in order to release RPE cells from the cell clusters. Lastly the solution containing released RPE cells and trypsin was added to CELLstart™ and collagen IV coated Ibidi wells (50 µl/cm^2). The cells were cultured at +37 ºC, and RPE DM- medium was changed twice per week. Cell attachment and growth were monitored under a stereomicroscope (Nikon, SMZ800, Nikon Instruments) and a phase contrast microscope (Eclipse TE2000-S, Nikon Instruments) for two weeks. RPE cell attachment and growth were classified as good, very good, low, or very low based on visual observations. Good RPE cell cluster attachment means that several hESC-RPE cells had attached to the wells. Very good RPE cell cluster attachment means that the attached hESC-RPE cells had formed colonies. Low RPE cell cluster attachment means that few hESC-RPE cells had attached to the wells. Very low RPE cell cluster attachment means that very few hESC-RPE cells had attached to the wells. Also in this case, the most important criterion in the

classification is the comparison of the cells and the colonies on different coating materials.

Two different coating concentrations of CELLstart™ and collagen IV were also tested in uncoated Ibidi wells. The cells used in this experiment were hESC-RPE cells differentiated from cell line Regea 08/023. Pigmented cell clusters were degraded enzymatically like described above, but the amount of added solution containing released RPE cells and trypsin to CELLstart™ and collagen IV coated Ibidi wells was smaller (25 µl/cm^2). Cell attachment and growth were monitored under a stereomicroscope (Nikon, SMZ800, Nikon Instruments) and a phase contrast microscope (Eclipse TE2000-S, Nikon Instruments) for one week. RPE cell attachment and growth were classified as good, very good, low, or very low based on visual observations, and the classification criteria were the same as in the previous paragraph.

Table 3.3. *Coating protocols in Confocal well test 2.*

Coating material	Coating concentration	Incubation	Cell line (Regea)	Degradation method	Biological replicates
CELLstart™	2.4 µl/cm²	2h, +37ºC	m 08/023	Mechanical	5
Collagen IV	5 µg/cm²	2h, +37ºC	m 08/023	Mechanical	5
CELLstart™	2.4 µl/cm²	2h, +37ºC	m 08/013	Enzymatic	8
Collagen IV	5 µg/cm²	2h, +37ºC	m 08/013	Enzymatic	8
CELLstart™	2.4 µl/cm²	2h, +37ºC	m 08/023	Enzymatic	4
CELLstart™ x 2	4.8 µl/cm²	2h, +37ºC	m 08/023	Enzymatic	4
Collagen IV	5 µg/cm²	2h, +37ºC	m 08/023	Enzymatic	4
Collagen IV x 2	10 µg/cm²	2h, +37ºC	m 08/023	Enzymatic	4

Abbreviation: m: mature, differentiated cells.

3.4. Cell culture in inserts

hESC-RPE cell attachment and growth were studied in BD BioCoat™ collagen IV cell culture inserts (BD Biosciences, Franklin Lakes, NJ, USA). bFGF was added to the culture medium to test if it accelerates the proliferation of hESC-RPE cells, or transdifferentiates them into neural retina cells or neurons. Inserts are small cups inside wells in a 24-well plate (Figure 3.2). At the bottom of the inserts there are track etched polyethylene terephthalate (PET) membranes which have pores of size 1.0 µm. The diameter of membrane size is 6.4 mm. Microporous membrane allows for the free diffusion of ions, low molecular weight lipoproteins, and other nutrients.

Figure 3.2. *Cell culture inserts (modified from Blanc-Labo SA 2010).*

Inserts were prepared for use according to the manufacturer's instructions. At first, each unit was re-hydrated by adding 250 µl of RPE DM- inside the insert, and incubated at RT for 30 minutes. After the incubation, RPE DM- medium was removed. 500 µl of fresh culture medium was added into the insert and to the well around the insert, although the medium should have been added only to the well according to the manufacturer's instructions. It was thought that the cells in the insert would miss culture medium if the medium was added only to the well. Three different culture mediums were used in the inserts and the wells around the inserts: RPE DM-, RPE DM- +5 ng/ml human bFGF (R&D Systems), and RPE DM- +10 ng/ml human bFGF.

hESC-RPE cells differentiated from cell line Regea 08/013 were used in the inserts. Pigmented cell clusters in suspension were cut to smaller pieces mechanically with a scalpel and a needle. 20-30 small pigmented cell clusters were passaged into each insert. Cells were cultured at +37 ºC, and the culture medium (RPE DM- or RPE DM- +5 ng/ml bFGF, or RPE DM- +10 ng/ml bFGF) was changed daily to the insert and to the well. Cell cluster attachment and growth were monitored under a stereomicroscope (Nikon, SMZ800, Nikon Instruments) and a phase contrast microscope (Eclipse TE2000-S, Nikon Instruments). The cell attachment and growth were classified as good, very good, or low based on visual observations. Good RPE cell cluster attachment means that the clusters had attached to the inserts. Very good RPE cell cluster attachment means that the attached clusters had grown to their surroundings. Low RPE cell cluster attachment means that the clusters had not attached to the inserts. Cell culture was carried on for two and a half weeks, after which the cells were used in Microelectrode Array (MEA) measurements in the thesis of Leena Lehtonen (Lehtonen 2010).

3.5. Cell characterization

3.5.1. Immunocytochemistry

Cells from Coating test 2, Confocal well test 1, and Confocal well test 2 were characterized by immunocytochemical stainings. The cells from Coating test 2 were stained with MITF, RAX, and PAX6, and the cells from Confocal well test 1 with bestrophin and CRALBP. Some of the cells from Confocal well test 2 were stained with bestrophin.

At first, the cells were washed twice with DPBS for 5 minutes. The cells were then fixed with 4 % paraformaldehyde in DPBS for 20 minutes at RT. The fixing was followed by washing twice with DPBS for 5 minutes. Fixed plates were stored at +4 ºC.

When performing the immunostaining, at first the cells were permeabilized and non-specific binding of the antibody was blocked with 10 % normal donkey serum (NDS), 0.1 % Triton® X-100 (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethyleneglycol), 1 % bovine serum albumin (BSA), all from Sigma-Aldrich, in DPBS for 45 minutes at RT. After blocking, the cells were washed with a solution of 1 % NDS, 0.1 % Triton® X-100, 1 % BSA, in DPBS for 5 minutes. Primary antibodies were diluted in a solution consisting of 1 % NDS, 0.1 % Triton® X-100, 1 % BSA in DPBS according to the Table 3.4, and incubated on the cells overnight at +4 ºC.

Table 3.4. *Primary antibodies used in immunocytochemistry.*

Primary antibody	Dilution	Origin of antibody	Manufacturer
MITF	1:100	Rabbit	Abcam PLC, Cambridge, UK
RAX	1:350	Rabbit	Abnova, Jhongli, Taiwan
PAX6	1:200	Mouse	DSHB, University of Iowa, USA
Bestrophin	1:250	Rabbit	Abcam PLC
CRALBP	1:400	Mouse	Abcam PLC

Next, the cells were washed three times with 1 % BSA in DPBS for 5 minutes. Secondary antibodies were diluted in 1 % BSA in DPBS according to the Table 3.5, and incubated on the cells in the dark at RT for one hour. The secondary antibody binds to the primary antibody. Unbound secondary antibodies were washed with DPBS (3 x 5 min) and phosphate buffer (PB) (2 x 5 min). The composition of PB is 27.6 g NaH_2PO_4, 28.6 g Na_2HPO_4, both from Sigma-Aldrich, and 1 l distilled water. Finally, the cells were mounted with Vectashield mounting medium containing 4´, 6´-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA), and covered with a cover slip. DAPI stains nuclei, and it can be detected in ultraviolet wavelength. Negative controls were labeled only with secondary antibodies.

Table 3.5. *Secondary antibodies used in immunocytochemistry.*

Secondary antibody	Dilution	Origin of antibody	Manufacturer
Alexa 568 IgG anti-mouse	1:800	Donkey	Molecular Probes, Invitrogen, Carlsbad, CA, USA
Alexa 488 IgG anti-rabbit	1:400	Donkey	Molecular Probes, Invitrogen
Alexa 568 IgG anti-rabbit	1:400	Goat	Molecular Probes, Invitrogen
Alexa 568 IgG anti-mouse	1:400	Goat	Molecular Probes, Invitrogen

The labeled cells from Coating test 2 and Confocal well test 1 were studied and photographed using Olympus IX51 phase contrast microscope with fluorescence optics and Olympus DP71-SET camera (Olympus Corporation, Tokyo). The labeled cells from Confocal well test 2 were studied and photographed using a confocal microscope (TCP SP2, Leica, Mannheim, Germany). Adobe Photoshop CS2 and CS4 and ImageJ were used for graphics.

3.5.2. RNA isolation

Total RNA was isolated from the cells of Coating test 2, in other words from the hESCs cultured on CELLstart™, Collagen IV, and Vitrocol™ coatings in RPE DM- medium. RNA isolation was performed from the cells in 24-well plates on three different time points: 7, 14, and 21 days with NucleoSpin® RNA XS-Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). There were two biological replicates of each time point for every coating material and cell line combination. hESCs cultured on three different coating materials were washed once with DPBS. Cells were detached from the bottom of the wells by twirling a pipette tip which was used upside down. The cells were collected and centrifuged at 13400 rpm for 60 seconds at RT. Cell pellet was resuspended into DPBS and centrifuged again. After the second centrifugation, the cell pellet was suspended into 100 µl Lysis Buffer RA1 (Macherey-Nagel) which inactivated RNases. Lysates were stored at -70 ºC until RNA was isolated. RNA isolation from the lysates was performed according to NucleoSpin® RNA XS-Kit instructions.

At first, Reducing Agent Tris(2-carboxyethyl)phosphine) (TCEP) was prepared according to the instructions, and 2 µl of TCEP was added to the lysate and vortexed vigorously. Lysis Buffer RA1 and TCEP homogenized the cultured cells. Carrier RNA working solution was prepared according to the instructions, and 5 µl of the working solution was added to the lysate and mixed by vortexing. The lysate was spun down briefly (1 s, 1000 x g) to clear the lid. Next, the lysate was filtered by centrifuging it through NucleoSpin® Filter (30 s, 11 000 x g). The filter was discarded, and 100 µl of 70 % ethanol was added to the homogenized lysate and mixed by pipetting up and down. RNA was bound to NucleoSpin® RNA XS Column by centrifuging the lysate (30 s, 11 000 x g). 100 µl of Membrane Desalting Buffer was added to the column and centrifuged (30 s, 11 000 x g) to dry the membrane. Salt removal made the following

rDNase digest more effective. rDNase reaction mixture was prepared according to the instructions, and 25 µl of the reaction mixture was added onto the silica membrane of the column and incubated for 15 minutes at RT. rDNase removed contaminating DNA from the lysate. Next, the silica membrane was washed and dried in three steps. In the first step, 100 µl of Wash Buffer RA2 was added to the column, incubated for 2 minutes at RT and centrifuged (30 s, 11 000 x g). Buffer RA2 inactivated the rDNase. In the second step, 400 µl of Wash Buffer RA3 was added to the column and centrifuged (30 s, 11 000 x g). In the third step, 200 µl of Wash Buffer RA3 was added to the column and centrifuged (2 min, 11 000 x g) to dry the membrane. The column was placed into a nuclease-free collection tube. Finally, the RNA was eluted in 14 µl RNase-free water (+ 37 ºC) and incubated for one minute. The sample was centrifuged (30 s, 11 000 x g) and transferred into ice. The concentrations and the qualities of isolated RNAs were determined by measuring absorbances at 260 nm and 280 nm with NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For pure RNA, the absorbance ratio (A260/280) should be close to two. RNA is thermodynamically instable, thus it was stored at –70 ºC.

3.5.3. Complementary DNA translation

cDNA was produced from the mRNA molecules by the enzyme reverse transcriptase. cDNA was synthesized from approximately 100 ng of the isolated total mRNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Reaction mixture contained 2 µl 10 x Buffer RT, 0.8 µl 25 x dNTP Mix (100 mM), 2 µl 10 x RT Random Primers, 1 µl MultiScribe™ Reverse Transcriptase, 1 µl RNase inhibitor (10 U/µl) in 1 x Buffer RT, and 100 ng isolated RNA. Sterile water was added to the mixture so that the final volume was 20 µl. RNase inhibitor was from Fermentas, Glen Burnie, MD, USA, and all other reagents from Applied Biosystems. The reaction mixture was incubated for 10 min at 25 ºC, 2 hours at 37 ºC, and 5 min at 85 ºC. Resulting cDNA was stored at -20 ºC, until it was used in q-RT-PCR.

3.5.4. Quantitative real time PCR

cDNAs from the cells of Coating test 2 were used in q-RT-PCR. The q-RT-PCR was performed with sequence-specific Applied Biosystems 20 x TaqMan Gene Expression Assays: MITF (Hs0111553_m1), RAX (Hs00429459_m1), PAX6 (Hs00240871_m1), SIX3 (Hs00193667_m1), OTX2 (Hs00222238_m1), and CHX10 (Hs01584048_m1). The expressions of MITF, RAX, and PAX6 genes were examined from both tested cell lines, and the expressions of SIX3, OTX2, and CHX10 were examined from Regea 06/040 cells. Normally MITF is expressed in mature RPE cells, RAX, PAX6, and OTX2 are expressed in neural progenitors, and SIX3 and CHX10 are expressed in photoreceptor progenitors. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 4352934-0803022) was used as a housekeeping control. Reaction mixture consisted of 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µl gene expression

assay, 1 µl translated cDNA, and 8 µl sterile water. Therefore the final volume of the reaction mixture was 20 µl. All samples and water controls were analyzed as triplicates. The q-RT-PCR was carried out with Applied Biosystems 7300 Real Time PCR System: 2 min at 50 ºC, 10 min at 95 ºC, and 40 cycles of 15 s at 95 ºC and 1 min at 60 ºC. Data was analyzed with 7300 System SDS Software (Applied Biosystems).

Samples' C_T values were determined with 7300 System SDS Software, and relative quantification was calculated with the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), also known as a comparative C_T method, which calculates relative changes in gene expressions. The data were normalized with the expression of the housekeeping gene GAPDH, and the expression levels of different genes in hESCs cultured on CELLstart™ were used as calibrators. Fold changes were calculated according to the equation:

Fold change =

$$2^{-[(C_{T,\,\text{target gene}} - C_{T,\,\text{GAPDH}}) - (C_{T,\,\text{CELLstart(Av)}} - C_{T,\,\text{GAPDH(Av)}})]}$$

Av is an abbreviation for average. If the fold change is greater or equal to 1, fold regulation is the same as the fold change. However, if the fold change is less than 1, the fold regulation is -1/(fold change). The gene expression difference between two samples is significant if the difference in fold regulation values is more than two. In this analysis, the fold regulations of the cells cultured on collagen IV and VitroCol™ were compared to the fold regulations of the cells cultured on CELLstart™. Microsoft Excel was used for graphical presentations.

4. RESULTS

4.1. Cell culture on different coating materials

Experiments with different coating materials consisted of two sections. In Coating test 1, especially the cell attachment and growth on different materials were observed, which was carried out by observing cells under a stereomicroscope and a phase contrast microscope daily. Based on the results from Coating test 1, the best coating materials were chosen for Coating test 2. In these further studies, the cell attachment and growth were observed again, but also cell differentiation was studied. The differentiation of hESCs towards RPE cells was examined by pigment observation, and from immunocytochemical staining and q-RT-PCR results.

4.1.1. Cell attachment and growth in Coating test 1

Cell attachment and growth results from Coating test 1 are presented in Table 4.1 and in Figure 4.1. The attachment of hESCs (Regea 08/013) cultured in RPE DM- medium was very good to CELLstart™ coating during the whole experiment (Figure 4.1.A-C). The cells formed colonies already on day two, and continued to grow well. Some of the cells detached during the experiment, but there were multiple cells and colonies in the wells all the time. CELLstart™ with additional proteins was not as good as CELLstart™ alone at the beginning of the experiment, but in the end all CELLstart™ coatings seemed to be almost equally good for the cell attachment and growth (Table 4.1). Accordingly, additional proteins did not enhance the cell attachment.

When classifying the cell attachment and growth in different time points, the most important criterion was the comparison of the attached cells and the colonies on different coating materials. Therefore the classifications between different time points are not completely comparable. For instance, CELLstart™+laminin had good cell attachment and growth on day two, low cell attachment and growth on day seven, and very good cell attachment and growth on day 14. On day seven, the cell attachment and growth on CELLstart™+laminin was worse than on CELLstart™+fibronectin, but on day 14, the cell attachment and growth on CELLstart™+laminin as good as on CELLstart™+fibronectin.

Cell attachment to collagen IV and VitroCol™ coatings was very good during the experiment. hESCs (Regea 08/013) cultured in RPE DM- medium formed rather big colonies on collagen IV coated wells in few days (Figure 4.1.D), and continued to grow well (Figure 4.1.E-F). As the experiment proceeded, the colonies became netlike. There were no remarkable differences in the cell attachment to three different collagen IV

coatings (collagen IV, collagen IV/CELLstart™, and collagen IV/fibronectin/ vitronectin/nidogen). Also on VitroCol™ coating, hESCs (Regea 08/013) cultured in RPE DM- medium were attached and formed colonies on day two (Figure 4.1.G), and continued to grow well (Figure 4.1.H-I). Some of the cells detached from collagen IV and VitroCol™ coatings during the experiment, but there were several cells and colonies in the wells throughout the experiment.

PEI/vitronectin coating did not support the cell attachment (Figure 4.1.M-N), as there were only few small colonies in the wells. The cell attachment to other PEI coatings (PEI with other additional proteins) was nearly similar throughout the experiment (Table 4.1). Some of the cells on PEI coatings were disgorged on day seven since the cell attachment was extremely weak. Caused by this, PEI/fibronectin, PEI/vitronectin, and PEI/laminin were not analyzed on day 14.

There were no remarkable differences in the cell attachment between two coating protocols for proteins ((1): mixing the protein with the basic coating material or (2): coating first the basic material and then the protein on the basic material). Therefore, only the former protocol was followed in poly-L-lysine coatings.

The attachment of hESCs (Regea 08/013) cultured in RPE DM- medium was low to all poly-L-lysine coatings during the whole experiment. There were only few attached cells and cell clusters which formed few small colonies (Figure 4.1.J-L). On day 14, there were practically no attached cells in poly-L-lysine/fibronectin coated wells.

Table 4.1. *Cell attachment and growth of hESCs (Regea 08/013) cultured in RPE DM-medium on different materials in Coating test 1. + -sign stands for good attachment and growth, ++ -sign very good attachment and growth, and - -sign low attachment and growth. Material/material stands for the first protein coating protocol, and material+material stands for the second protein coating protocol.*

Coating material	hESC cluster attachment (d2)	Cell growth (d7)	Cell growth (d14)
CELLstart™	++	++	++
CELLstart™/fibronectin	+	+	++
CELLstart™+fibronectin	+	+	++
CELLstart™/vitronectin	-	+	++
CELLstart™+vitronectin	-	++	++
CELLstart™/laminin	-	-	+
CELLstart™+laminin	+	-	++
Collagen IV	++	++	++
Collagen IV/CELLstart™	++	++	++
Collagen IV/fibron./vitron./nidogen	++	++	++
VitroCol™	++	++	++
Poly-L-lysine/fibronectin	-	-	-
Poly-L-lysine/vitronectin	-	-	-
Poly-L-lysine/laminin	-	-	-
PEI/fibronectin	-	-	n.a.
PEI+fibronectin	-	+	-
PEI/vitronectin	-	-	n.a.
PEI+vitronectin	-	+	-
PEI/laminin	-	-	n.a.
PEI+laminin	-	-	-

Abbreviations: d: day; fibron: fibronectin; vitron: vitronectin; n.a.: not analyzed (experiment had been finished).

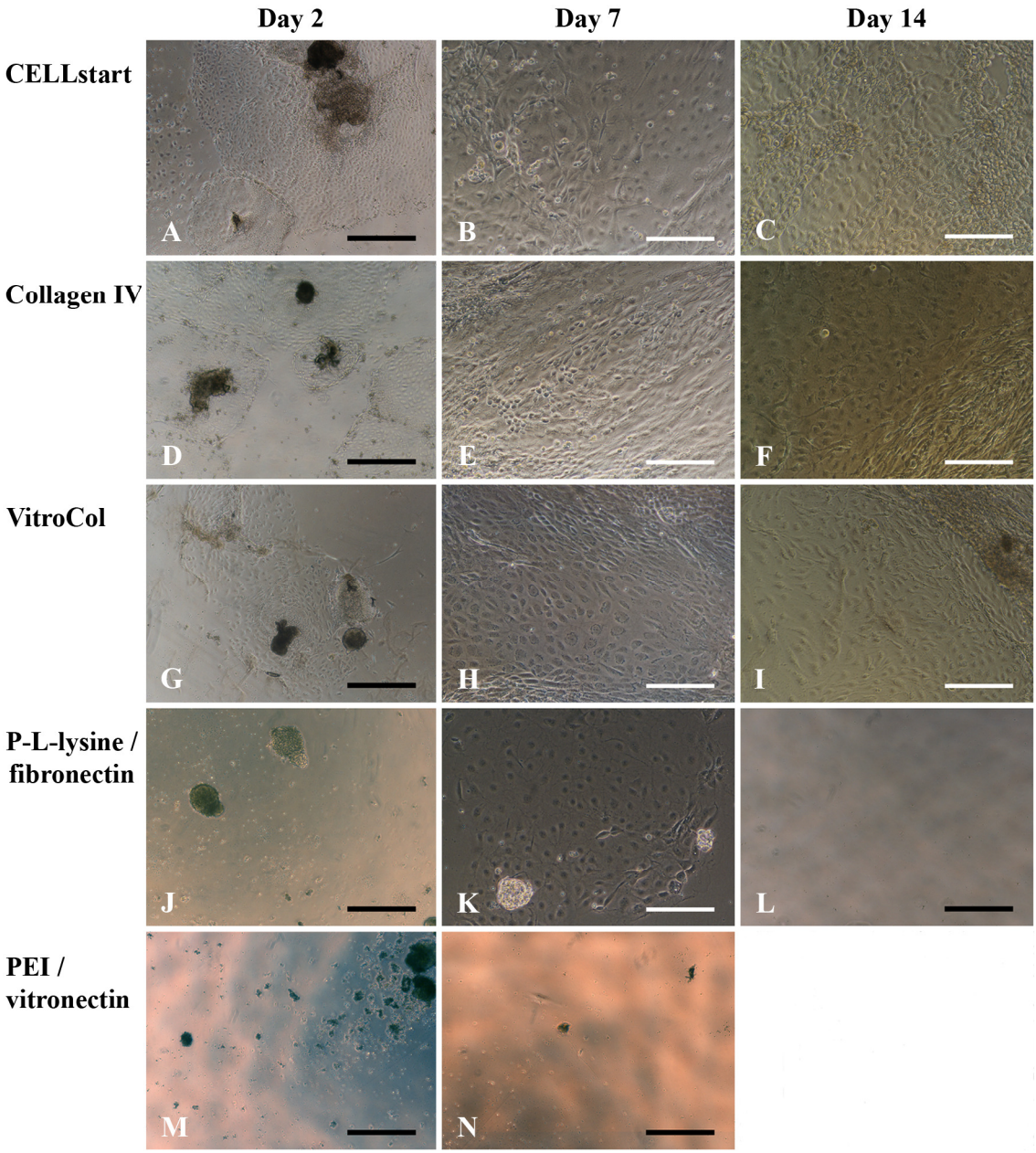

Figure 4.1. *hESCs (Regea 08/013) cultured in RPE DM- medium. Coating materials are presented on the left and the time points above the picture. P-L-lysine stands for poly-L-lysine. Black scale bar 500 µm, white scale bar 200 µm.*

4.1.2. Cell attachment and growth in Coating test 2

The best coating materials from Coating test 1 were chosen for further studies in Coating test 2. CELLstart™, collagen IV, collagen IV/CELLstart™, collagen IV/ fibronectin/vitronectin/nidogen, and VitroCol™ supported the attachment of hESCs (Regea 08/013) cultured in RPE DM- medium equally well (Table 4.1). However, since collagen IV/CELLstart™ and collagen IV/fibronectin/vitronectin/nidogen were not superior to collagen IV alone, and they more laborious to use than collagen IV, they were not chosen for further studies. Therefore CELLstart™, collagen IV, and VitroCol™ were chosen for Coating test 2 with two cell lines. In these further studies,

the cell attachment to three tested materials was almost equally good during the experiment (Table 4.2), as well as in Coating test 1. Cell lines Regea 06/040 and Regea 08/013 did not have remarkable differences in the cell attachment. Coating test 2 was continued to day 21 when there were still big colonies in the wells (Figure 4.2.A-C). However, cavities appeared in some colonies. Cavities in Regea 06/040 cell colonies on VitroCol™ coating and in Regea 08/013 cell colonies on collagen IV coating were noticed on day 17. Cavities were noticed also in Regea 08/013 colonies on VitroCol™ coating on day 21 (Figure 4.2.C). In addition, some cells detached from every coating material and cell line combination during the experiment. Cell detachment increased at the end of the test. Nevertheless, very good cell attachment and big colonies were detected throughout the experiment.

Table 4.2. *Cell attachment and growth of hESCs (Regea 06/040 and Regea 08/013) cultured in RPE DM- medium on different materials in Coating test 2. + -sign stands for good attachment and growth, and ++ -sign very good attachment and growth.*

Coating material	Cell line (Regea)	hESC cluster attachment (d2)	Cell growth (d7)	Cell growth (d14)	Cell growth (d21)
CELLstart™	06/040	++	++	++	++
CELLstart™	08/013	++	+	++	++
VitroCol™	06/040	+	++	++	++
VitroCol™	08/013	++	++	++	++
Collagen IV	06/040	++	++	++	++
Collagen IV	08/013	++	++	++	++

Abbreviation: d: day.

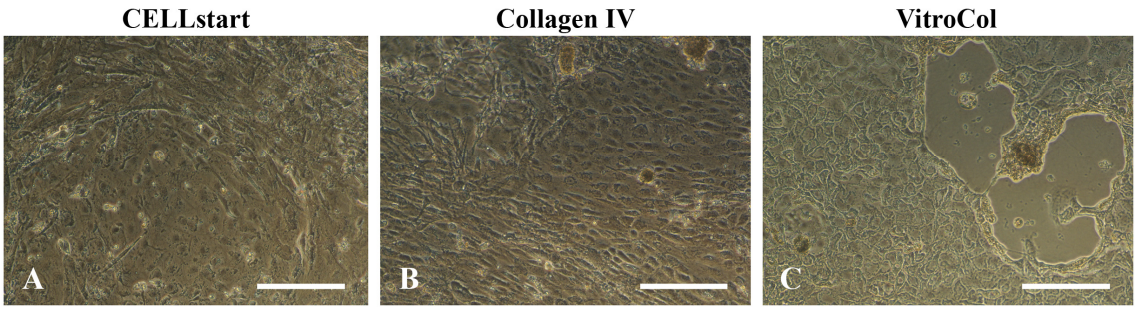

Figure 4.2. *hESCs (Regea 08/013) cultured in RPE DM- medium for 21 days on three coating materials. Scale bar 200 µm.*

Cell pigmentation was observed. On day 17, the first observation of the pigmentation was made visually. Regea 06/040 cell colonies had pigmented areas on every tested coating material (CELLstart™, collagen IV, and VitroCol™). Regea 08/013 cell colonies had pigmented areas on CELLstart™ and VitroCol™ coatings.

Nevertheless, the colonies were still mainly white since pigmented brown areas covered less than 10 % of the colonies.

4.1.3. Cell differentiation by immunocytochemistry

The cell differentiation of hESCs (Regea 06/040 and Regea 08/013) cultured in RPE DM- medium on different coating materials towards RPE cells was evaluated by immunocytochemical stainings. The cells from Coating test 2 were stained with MITF, RAX, and PAX6 antibodies. DAPI was used to stain nuclei.

During the immunocytochemical staining, some of the samples partly detached, and therefore layers were formed to the samples. Due to the layers, single cells were rather difficult to distinguish. Thus the staining results were relatively unclear. Nevertheless, the cells cultured on CELLstart™, collagen IV, and VitroCol™ for 21 days seemed to be slightly positive against MITF, a marker for RPE cells. This was noticed from immunicytochemical stainings, as the cells slightly expressed MITF marker. MITF is a transcription factor, so it is expressed in the nuclei. However, transcription factor antibodies can usually be detected all around the cell in immunocytochemical stainings, like in Figure 4.3.A. The cells on all three coating materials seemed to express also neural progenitor markers RAX and PAX6 (data not shown). As well as MITF, RAX is a transcription factor, and the expression of RAX can be perceived in Figure 4.3.B.

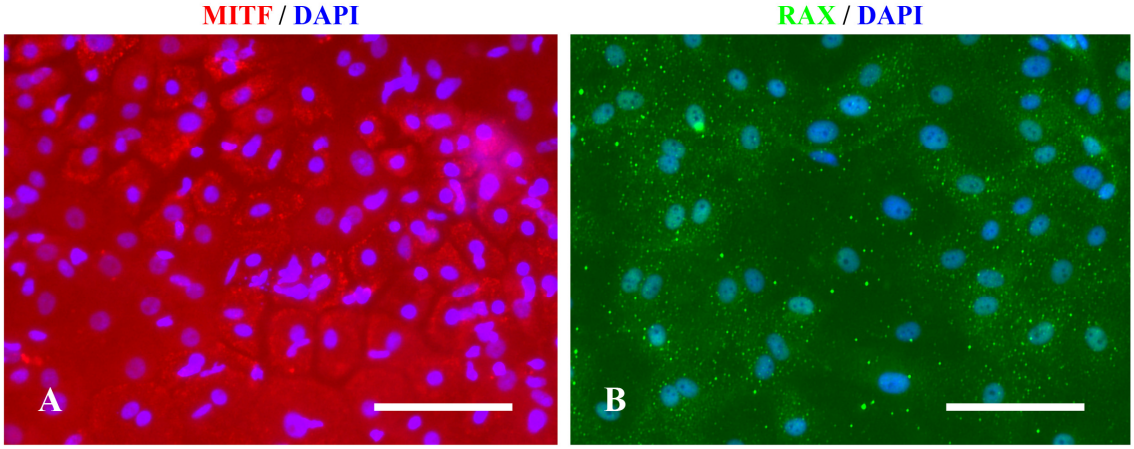

Figure 4.3. *Immunostaining of hESCs (Regea 08/013) cultured in RPE DM- medium and on VitroCol™ coating for 21 days. Stainings: A) MITF and DAPI, B) RAX and DAPI. Scale bar 100µm.*

4.1.4. Cell differentiation by quantitative real time PCR

The cell differentiation of the hESCs cultured in RPE DM- medium on different coating materials towards RPE cells was evaluated also by q-RT-PCR. Fold regulations of the cells cultured on collagen IV and VitroCol™ were compared to those of the cells cultured on CELLstart™. Analyzed cells were Regea 06/040 and Regea 08/013 cultured in RPE DM- medium in Coating test 2. Mean fold regulations in gene expressions are presented in Figures 4.4-4.9. Standard deviations of the three experimental replicates are

presented as error bars. An asterisk round a bar means that the gene expression difference is significant compared to the gene expression of the cells cultured on CELLstart™. If there are two biological replicates, asterisks are marked only if both replicates have significant differences, and the differences are not controversial. The controversial difference means that the expression of a certain gene is significantly higher in one biological replicate and significantly lower in the other biological replicate compared to the gene expression of the cells cultured on CELLstart™.

On day seven, the expressions of RAX, PAX6, SIX3, and OTX2 were significantly lower in Regea 06/040 cells cultured on collagen IV than on CELLstart™. On the contrary, the expression of CHX10 was significantly higher in Regea 06/040 cells cultured on collagen IV compared to the cells cultured on CELLstart™. The expressions of RAX, PAX6, and OTX2 were significantly lower in Regea 06/040 cells cultured on VitroCol™ than on CELLstart™ on day seven. On day 21, the expressions of RAX, PAX6, SIX3, OTX2, and CHX10 were significantly lower in Regea 06/040 cells cultured on collagen IV or VitroCol™ than on CELLstart™. Other differences in gene expressions for Regea 06/040 cells were either not significant or they were controversial between two biological replicates.

On day seven, the expressions of RAX and PAX6 were significantly lower in Regea 08/013 cells cultured on collagen IV than on CELLstart™. On day 14, the expression of MITF was significantly lower in Regea 08/013 cells cultured on collagen IV or on VitroCol™ than on CELLstart™. Other differences in gene expressions for Regea 08/013 cells were either not significant or they were controversial between two biological replicates.

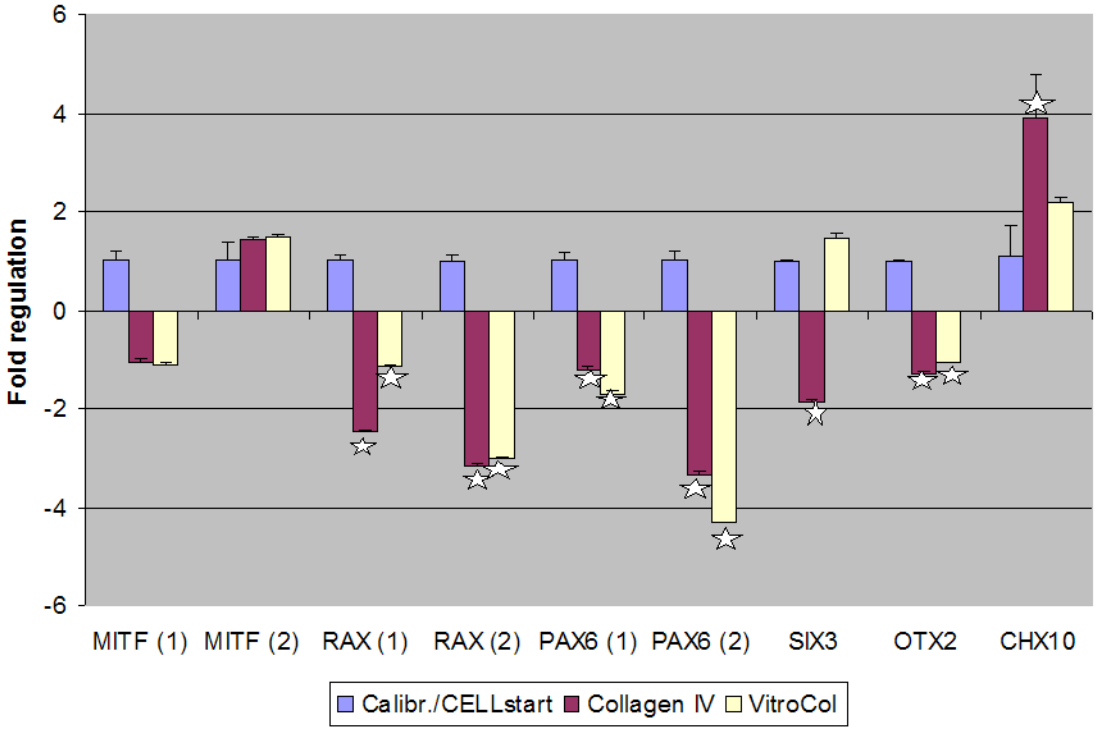

Figure 4.4. *Mean fold regulations in MITF, RAX, PAX6, SIX3, OTX2, and CHX10 expressions for hESCs (Regea 06/040) cultured in RPE DM- medium and on CELLstart™, collagen IV, or VitroCol™ coating for seven days. Samples (1) and (2) are biological replicates.*

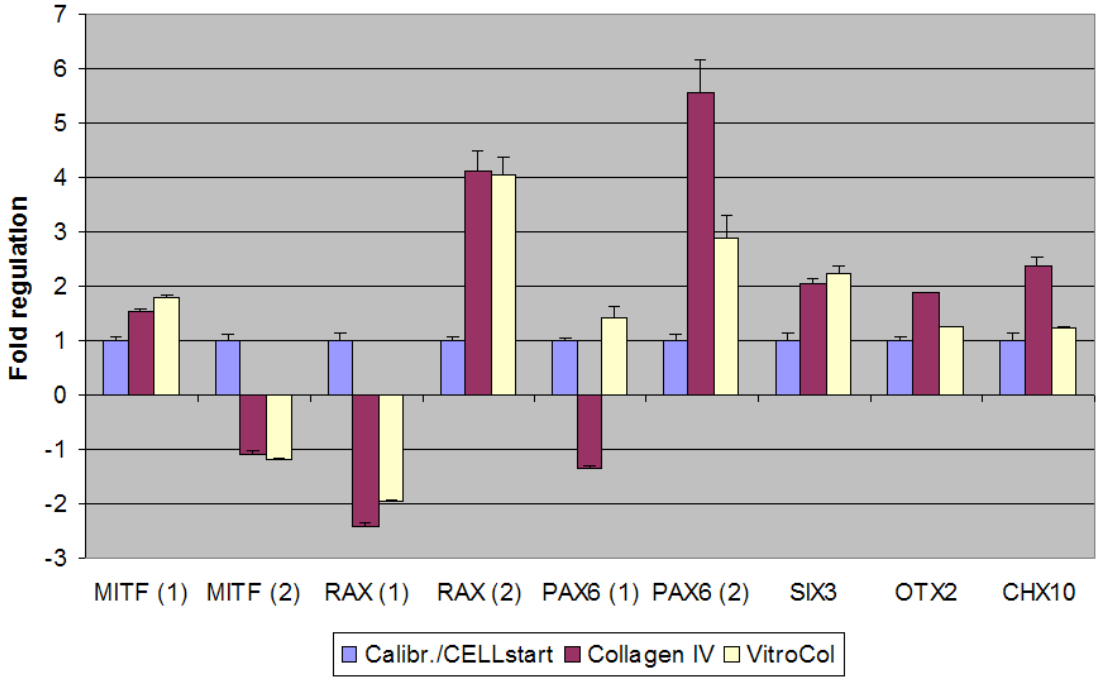

Figure 4.5. *Mean fold regulations in MITF, RAX, PAX6, SIX3, OTX2, and CHX10 expressions for hESCs (Regea 06/040) cultured in RPE DM- medium and on CELLstart™, collagen IV, or VitroCol™ coating for 14 days. Samples (1) and (2) are biological replicates.*

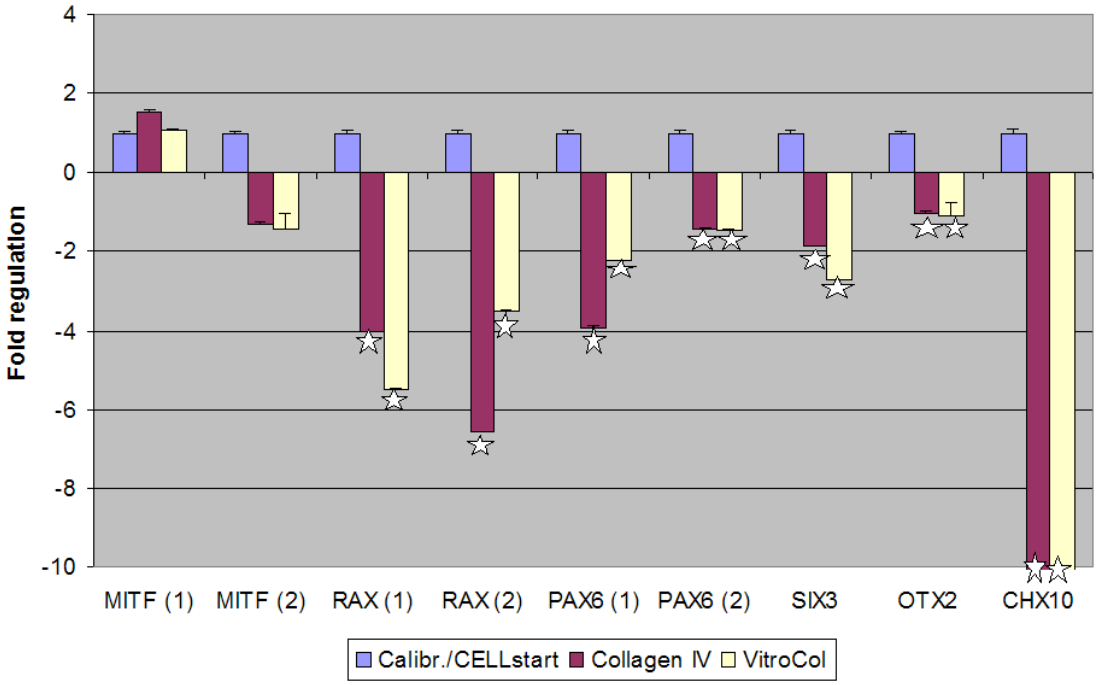

Figure 4.6. *Mean fold regulations in MITF, RAX, PAX6, SIX3, OTX2, and CHX10 expressions for hESCs (Regea 06/040) cultured in RPE DM- medium and on CELLstart™, collagen IV, or VitroCol™ coating for 21 days. Samples (1) and (2) are biological replicates.*

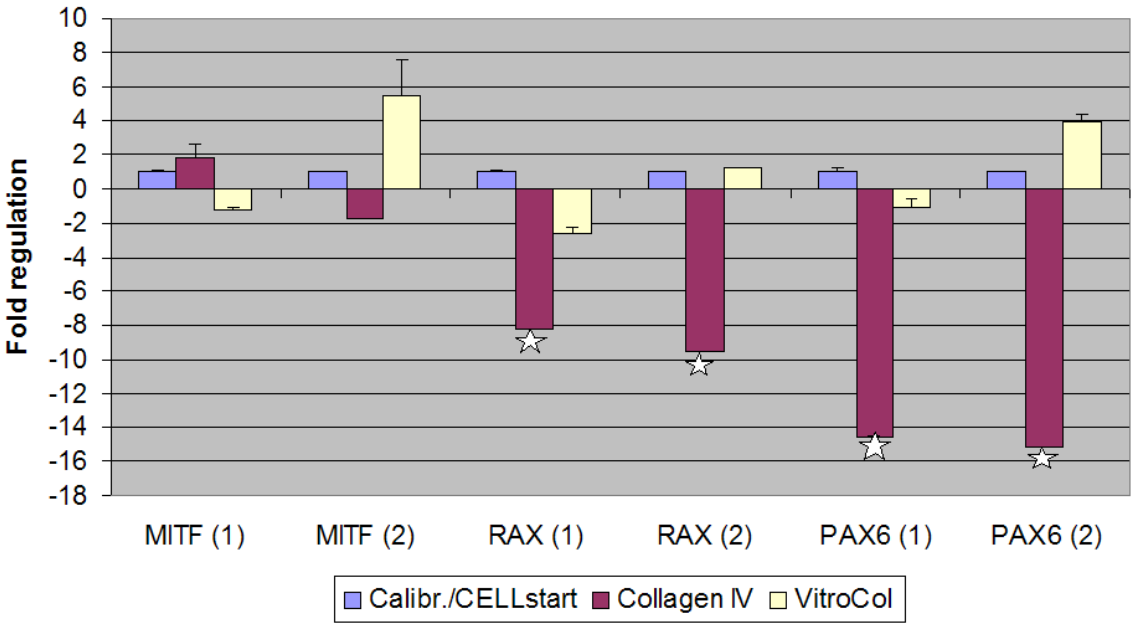

Figure 4.7. *Mean fold regulations in MITF, RAX, and PAX6 expressions for hESCs (Regea 08/013) cultured in RPE DM- medium and on CELLstart™, collagen IV, or VitroCol™ coating for seven days. Samples (1) and (2) are biological replicates.*

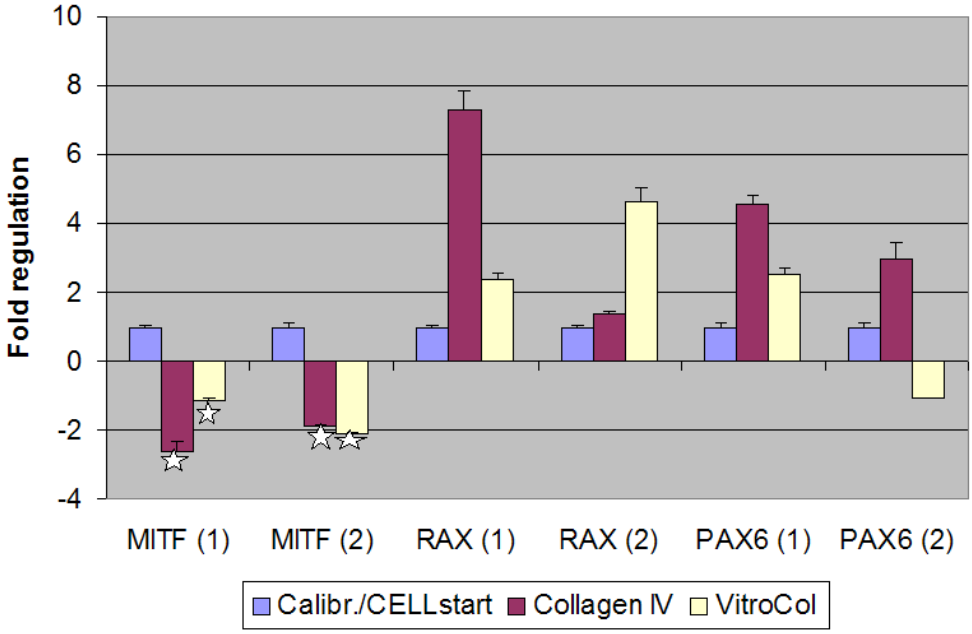

Figure 4.8. *Mean fold regulations in MITF, RAX, and PAX6 expressions for hESCs (Regea 08/013) cultured in RPE DM- medium and on CELLstart™, collagen IV, or VitroCol™ coating for 14 days. Samples (1) and (2) are biological replicates.*

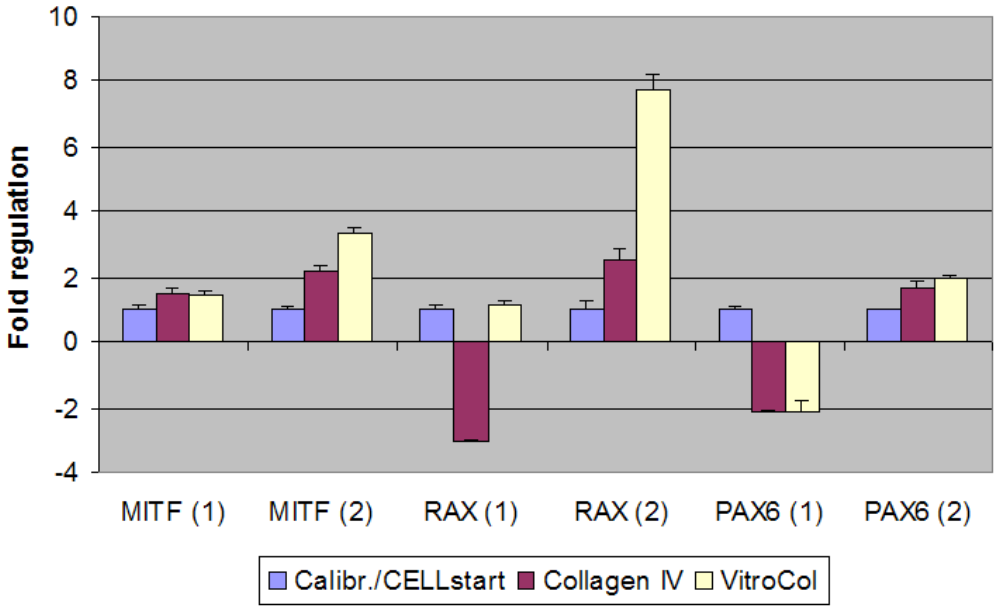

Figure 4.9. *Mean fold regulations in MITF, RAX, and PAX6 expressions for hESCs (Regea 08/013) cultured in RPE DM- medium and on CELLstart™, collagen IV, or VitroCol™ coating for 21 days. Samples (1) and (2) are biological replicates.*

4.2. Confocal microscopy wells

Cell attachment and growth in commercially coated and self-coated confocal microscopy wells were observed visually under a microscope. At first, the cell attachment of hESCs cultured in RPE DM- medium to the commercially coated Ibidi wells was tested in Confocal well test 1. The cell differentiation was evaluated by pigment observation and immunocytochemical staining results. Secondly, the cell

attachment of hESC-RPE cells to the self-coated Ibidi wells was tested in Confocal well test 2.

4.2.1. Confocal well test 1

The results of the experiments in Confocal well test 1 are presented in Table 4.3. The attachment of hESCs cultured in RPE DM- medium was better to collagen IV Ibidi wells than to ibiTreat or poly-L-lysine Ibidi wells. During the experiment, there were no remarkable differences in the cell attachment and growth between two tested cell lines (Regea 06/040 and Regea 08/013). On the contrary, the effects of the coating materials on the cell attachment could be noticed already on the first days of the test. Cell clusters attached to collagen IV Ibidi wells on day one, and started to form colonies (Figure 4.10.A). Black arrows in Figure 4.10.A indicate the edges of the colony. At that time, there were no attached cell clusters in ibiTreat Ibidi wells and only few in poly-L-lysine Ibidi wells (Figure 4.10.B-C). On day four, few cell clusters had attached to ibiTreat Ibidi wells, but some attached cell clusters in poly-L-lysine Ibidi wells were forming colonies. At that time, the colonies in collagen IV coated wells continued to grow well. On day eight, the differences in colony formation were clear between three Ibidi coatings. Collagen IV Ibidi wells had big colonies, whereas ibiTreat and poly-L-lysine Ibidi wells had only small and thin colonies (Figure 4.10.D-F). By the day 10, collagen IV Ibidi wells were almost confluent. Even though the differences between three tested Ibidi coatings seemed to be obvious, there were remarkable variations between biological replicates. For instance, on day 10, there was one rather big Regea 06/040 colony in one ibiTreat Ibidi well, but other three ibiTreat Ibidi wells with the same cell line were almost empty.

As the experiment proceeded, ibiTreat Ibidi wells seemed to support the cell attachment and growth slightly better than poly-L-lysine Ibidi wells. On day 15, there were some rather small cell colonies in ibiTreat Ibidi wells, whereas in poly-L-lysine Ibidi wells there were mainly only few attached cell clusters. On day 18, two ibiTreat Ibidi Regea 06/040 wells, two poly-L-lysine Regea 06/040 wells, and one poly-L-lysine Regea 08/013 wells were emptied since there were extremely few growth. At that time, there were still big colonies in collagen IV Ibidi wells, although some cells were detaching. On day 21, the cell colonies in collagen IV Ibidi wells partly detached from the wells. Small colonies or cell clusters in ibiTreat and poly-L-lysine Ibidi wells detached also partly, but not as much as the colonies in collagen IV Ibidi wells.

Table 4.3. *Cell attachment and growth of hESCs (Regea 06/040 and Regea 08/013) cultured in RPE DM- medium in Ibidi confocal microscopy wells. + -sign stands for good attachment and growth, ++ -sign very good attachment and growth, - -sign low attachment and growth, and -- -sign very low attachment and growth.*

Ibidi coating	Cell line (Regea)	hESC cluster attachment (d1)	Cell growth (d4)	Cell growth (d8)	Cell growth (d15)	Cell growth (d21)
Collagen IV	06/040	+	++	++	++	++
Collagen IV	08/013	+	++	++	++	++
IbiTreat	06/040	--	-	-	-	-
IbiTreat	08/013	--	-	-	-	+
Poly-L-lysine	06/040	+	+	-	--	--
Poly-L-lysine	08/013	-	+	-	--	--

Abbreviation: d: day.

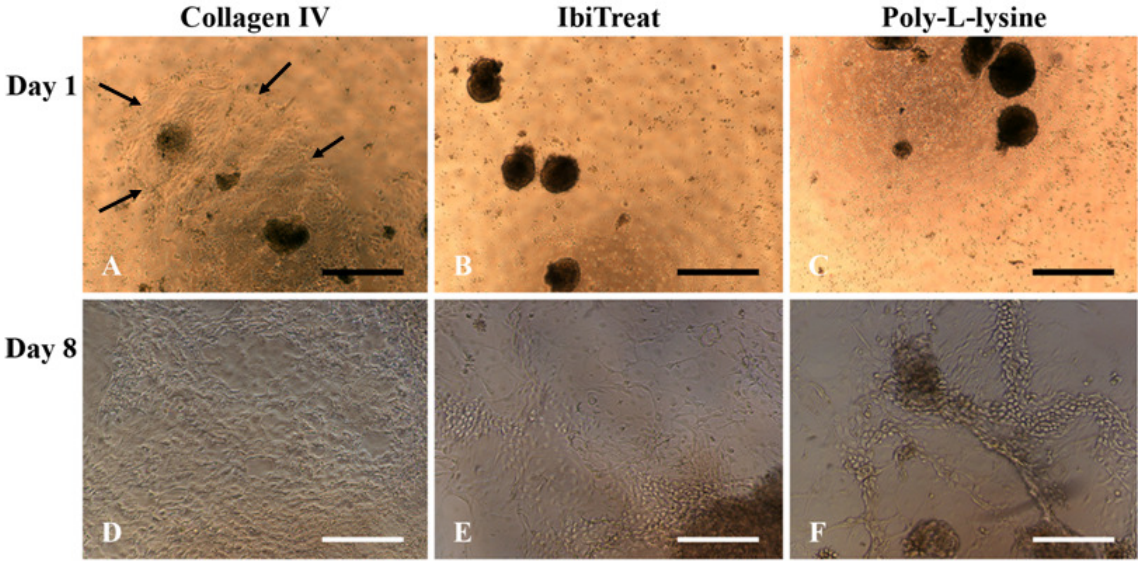

Figure 4.10. *hESCs (Regea 08/013) cultured in RPE DM- medium and in Ibidi confocal microscopy wells. Tested commercial coatings are presented above the picture and the time points are on the left. Black scale bar 500 µm, white scale bar 200 µm.*

Cell pigmentation was observed. Pigmentation was noticed at the end of the experiment in Regea 06/040 and Regea 08/013 colonies in collagen IV Ibidi wells, but not in ibiTreat or poly-L-lysine Ibidi wells. In spite of the pigmentation on collagen IV Ibidi wells, the colonies were still mainly white since brown pigmented areas covered less than 10 % of the colonies.

Cell characterization from Confocal well test 1 samples by immunocytochemistry was practically unfeasible. Since many of the colonies started to detach before the staining and also during the staining, the samples were on layers. Therefore it was rather

impossible to distinguish single cells and interpret if they were positive for bestrophin and CRALPB or not.

4.2.2. Confocal well test 2

The best coating material from Confocal well test 1, collagen IV, was tested also in uncoated Ibidi wells in Confocal well test 2. Cell attachment to self-coated collagen IV Ibidi wells was compared to self-coated CELLstart™ Ibidi wells. The results of this experiment are presented in Table 4.4. Mechanically cut RPE cell clusters had low attachment to both tested coating materials throughout the experiment. hESC-RPE cells used in this experiment were originally differentiated from cell line Regea 08/023. By the day seven, about one cell cluster was attached per collagen IV self-coated Ibidi well, but they had not grown. Cell attachment was slightly worse to CELLstart™ self-coated Ibidi wells. On day 14, most of the cell clusters were unattached on both coatings, and no colony formation was noticed (Figure 4.11). As the experiment proceeded, there were no remarkable changes in the cell attachment. Few more cell clusters attached to collagen IV self-coated Ibidi wells than to CELLstart™ self-coated Ibidi wells, and some cell clusters detached from the CELLstart™ self-coated Ibidi wells.

Table 4.4. *Cell attachment and growth of hESC-RPE cells in self-coated Ibidi confocal microscopy wells. x 2 -sign after coating material stands for the double concentration of the coating material. + -sign stands for good attachment and growth, ++ -sign very good attachment and growth and - -sign low attachment and growth, and -- -sign very low attachment and growth.*

Coating material	Cell line (Regea)	Degradation method	RPE cell cluster attachment (d1)	RPE cell cluster attachment (d7)	RPE cell cluster attachment (d14)	RPE cell cluster attachment (d21)
CELLstart™	m 08/023	Mechanical	-	--	--	--
Collagen IV	m 08/023	Mechanical	-	-	-	-
CELLstart™	m 08/013	Enzymatic	+	+	+	n.a.
Collagen IV	m 08/013	Enzymatic	+	++	++	n.a.
CELLstart™	m 08/023	Enzymatic	+	+	n.a.	n.a.
CELLstart™ x 2	m 08/023	Enzymatic	+	+	n.a.	n.a.
Collagen IV	m 08/023	Enzymatic	+	++	n.a.	n.a.
Collagen IV x 2	m 08/023	Enzymatic	+	+	n.a.	n.a.

Abbreviations: d: day; m: mature, differentiated cells; n.a.: not analyzed (experiment had been finished).

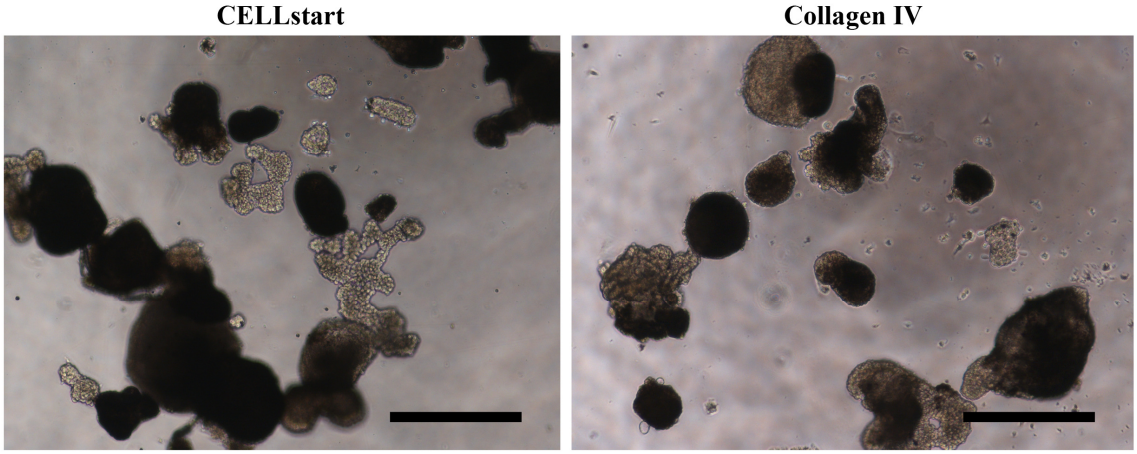

Figure 4.11. *Mechanically cut RPE cell clusters (Regea 08/023) in CELLstart™ and collagen IV self-coated Ibidi confocal microscopy wells on day 14. Scale bar 500 µm.*

hESC-RPE cells derived from enzymatic degradation attached better to self-coated Ibidi confocal microscopy wells than mechanically cut small RPE cell clusters (Table 4.4). The hESC-RPE cells used in this experiment were originally differentiated from cell line Regea 08/013. On the day subsequent to the passage, several cells had already attached to the self-coated Ibidi wells although there were many unattached cells. At this point, the cell attachment to CELLstart™ and collagen IV self-coated Ibidi wells was equally good (Figure 4.12.A, D). On day four, there were more attached cells in collagen IV self-coated Ibidi wells than in CELLstart™ self-coated Ibidi wells. Attached cells formed some cell clusters in both well types. On day seven, the cells in collagen IV self-coated Ibidi wells had formed some colonies (Figure 4.12.E), unlike in CELLstart™ self-coated Ibidi wells (Figure 4.12.B). At the end of this experiment, there were several attached hESC-RPE cell clusters in CELLstart™ self-coated Ibidi wells, but they had not grown (Figure 4.12.C). On the other hand, there were cell colonies in collagen IV self-coated Ibidi wells (Figure 4.12.F). However, the colonies also contained other cells than RPE cells since there were great non-pigmented areas. The cells cultured in self-coated collagen IV Ibidi wells expressed bestrophin which is a cell membrane protein (Figure 4.13). The picture was taken using a confocal microscope.

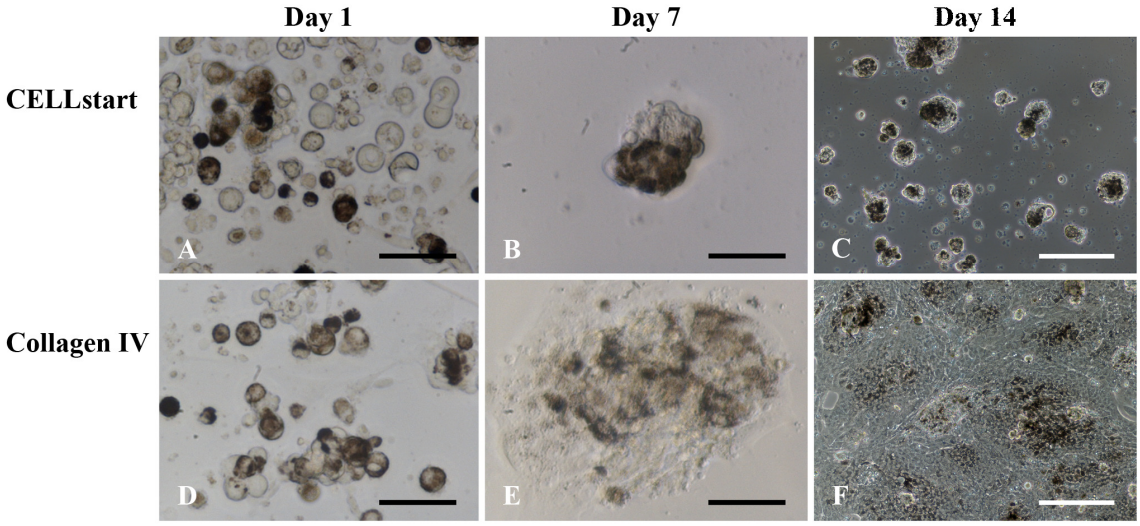

Figure 4.12. *Enzymatically degraded RPE cell clusters (Regea 08/013) in CELLstart™ and collagen IV self-coated Ibidi confocal microscopy wells. Time points are presented above the picture. Black scale bar 50 µm, white scale bar 200 µm.*

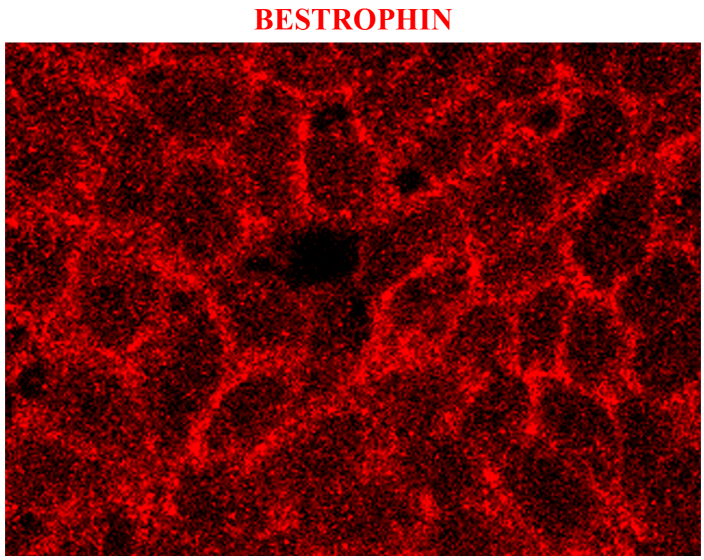

Figure 4.13. *Immunostaining of hESCs-derived (Regea 08/013) RPE cells cultured in collagen IV self-coated Ibidi confocal microscopy well for 15 days. Staining was bestrophin.*

The effect of the concentration of the coating material was also tested with self-coated Ibidi confocal microscopy wells. hESC-RPE cells derived from the enzymatic degradation were passaged to CELLstart™ and collagen IV coated wells with normal and double concentrations. The normal concentrations of CELLstart™ and collagen IV coatings were 2.4 µl/cm^2 and 5 µg/cm^2, and the double concentrations were 4.8 µl/cm^2 and 10 µg/cm^2, respectively. The attachment of the hESC-RPE cells to normally self-coated CELLstart™ and collagen IV Ibidi wells was the same as in the previous experiment. The hESC-RPE cells used in this experiment were originally differentiated from cell line Regea 08/023, as they were differentiated from Regea 08/013 in the previous experiment. Therefore, the original cell line did not seem to have an effect on

the cell attachment. The double concentration of the coating material did not support the cell attachment better than the normal concentration of the coating material (Table 4.4). Actually, there seemed to be slightly less attached cells in the wells with the double concentration of the coating material throughout the experiment.

4.3. Cell culture in inserts

Cell attachment to Collagen IV Cell Culture Inserts was relatively difficult to notice. When hESC-RPE cell clusters, originally differentiated from Regea 08/013, were observed under a microscope and the plate was moved, it was challenging to detect if the inserts were only moving, or both the inserts and the cell clusters. On the second day, the cell clusters seemed to be unattached in all tested culture conditions (RPE DM- or RPE DM- +5 ng/ml bFGF, or RPE DM- +10 ng/ml bFGF) (Table 4.5; Figure 4.14.A-C). On day eight, it seemed that the cell clusters were attached, but they had not grown. At this point, the cell attachment was equally good in all three wells, so culture medium did not seem to affect the cell attachment. On day 14, there seemed to be more cell clusters in two inserts containing bFGF in their culture medium than in the insert with RPE DM- as a culture medium. No cell clusters were added or removed from the inserts, so the cell clusters must have divided in the two inserts containing bFGF in their culture medium. Nonetheless, the differences in the amounts of the cell clusters between three different culture conditions were not remarkable. In all three inserts, attached cell clusters had grown little to their surroundings at this point (Figure 4.14.D-F), and the grown areas had RPE cell appearance. On day 17, the pigmented cell clusters were still attached, and had grown. In addition, there were no remarkable differences in the cell attachment or growth between three insert conditions. Visual observation could not verify the transdifferentiation of the hESC-RPE cells into neural retina cells or neurons at any time point of the experiment.

Table 4.5. *Cell attachment and growth of hESC-RPE cells in Collagen IV Cell Culture Inserts. + -sign stands for good attachment and growth, ++ -sign very good attachment and growth, and - -sign low attachment and growth.*

Culture medium	Cell line (Regea)	Degradation method	RPE cell cluster attachment (d1)	RPE cell cluster attachment (d8)	RPE cell cluster attachment (d14)
RPE DM-	m 08/013	Mechanical	-	+	++
RPE DM- +5 ng/ml bFGF	m 08/013	Mechanical	-	+	++
RPE DM- +10 ng/ml bFGF	m 08/013	Mechanical	-	+	++

Abbreviations: d: day; m: mature, differentiated cells.

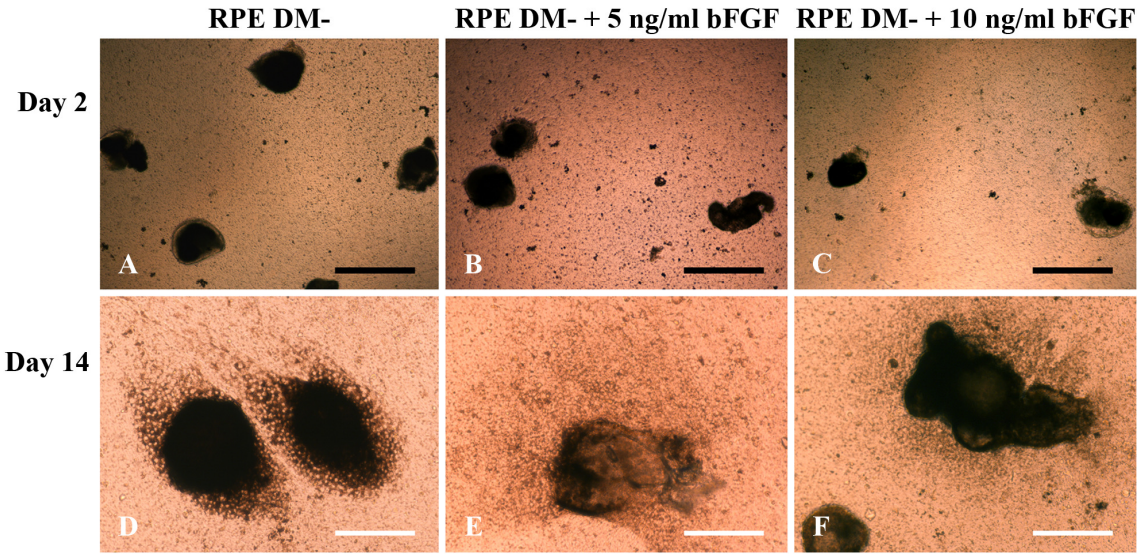

Figure 4.14. *Mechanically cut RPE cell clusters (Regea 08/013) in Collagen IV Cell Culture Inserts. Time points are presented on the left and culture mediums above the picture. Black scale bar 500 µm, white scale bar 200 µm.*

5. DISCUSSION

Cell-material interactions on 2D-coating materials have not been very largely studied, even though these interactions may affect cell properties, and coating materials are widely used in cell culture. Coating materials can assist cell attachment in adherent cultures, and they can also affect cell differentiation.

The aim of this thesis was to evaluate the cell attachment and differentiation of hESC-RPE cells on different coating materials and culture conditions. The project consisted of three different parts which are discussed here separately. hESCs were derived and maintained without serum and animal feeder cells. In all experiments, the goal was to improve culture conditions for hESC-RPE cells, and therefore promote RPE research.

5.1. Cell culture on different coating materials

The aim of this first study was to evaluate the effects of xeno-free coating materials on cell attachment and differentiation. Cell culture lasted for 14-21 days, and the only factors affecting hESC (Regea 06/040 and Regea 08/013) differentiation towards RPE cells were coating materials and culture medium lacking bFGF. CELLstart™, collagen IV, and VitroCol™ supported the cell attachment and growth better than other tested coating materials (poly-L-lysine and PEI with additional proteins fibronectin, vitronectin, and laminin; material combinations are presented in Table 3.1). There were no remarkable differences in the cell attachments to CELLstart™, collagen IV, and VitroCol™ coatings. In addition, the differentiation of hESCs towards RPE cells, analyzed by visual pigment observation, immunocytochemistry, and q-RT-PCR, seemed to be nearly equally good on these three coating materials.

To my knowledge, no publication of hESC differentiation towards RPE cells with a xeno-free coating material and a serum-free culture medium in adherent cell culture exists. Therefore there are no studies comparable to this one. Klimanskaya and co-workers (2004) and Klimanskaya (2006) have succeeded in adherent hESC differentiation towards RPE cells on gelatin, fibronectin, laminin, collagen I, and collagen IV, but they used MEF feeders and FBS in culture medium. MEF feeders have been reported to support RPE differentiation (reviewed by Vugler et al. 2007) and FBS promotes cell adhesion and growth (Klauser et al. 2010). Adherent culture conditions lacking these factors are more challenging.

Nevertheless, if the results of this study are evaluated, there are similarities to previous studies. Collagen IV and VitroCol™ (97 % of which is collagen I) supported the cell attachment, parallel to Klimanskaya and co-workers' (2004) and Klimanskaya's

(2006) results. The attachment and differentiation results concerning VitroCol™ are not completely reliable since VitroCol™ became unsterile in the neutralization procedure. It was expected that at the beginning of the experiment hESCs would attach well to CELLstart™ coating, since CELLstart™ supports hESC attachment according to the manufacturer. As the experiment proceeded, and the hESCs differentiated, the cells continued to be attached to CELLstart™ coating, which was slightly unexpected. However, it is rather difficult to estimate the effects of CELLstart™ coating material because its composition is partly unknown. To my knowledge, poly-L-lysine and PEI have not been used in similar experiments, and therefore it was interesting to test how these coating materials would support the cell attachment and differentiation. In addition, it was interesting to test if poly-L-lysine would affect similarly as poly-D-lysine which has been successfully used in hESC-RPE culture with laminin and fibronectin (Osakada et al. 2008; Idelson et al. 2009). The effect of poly-L-lysine differed from that of poly-D-lysine, since the cell attachment to poly-L-lysine and PEI was so weak that the differentiation could not be evaluated. Nevertheless, Osakada and co-workers' culture conditions contained MEFs and EBs and Idelson and co-workers' culture conditions contained EBs, so the results of these publications are not comparable to this study.

It was also interesting that the combinations of additional proteins and basic materials were not superior to basic materials alone. Fibronectin, vitronectin, and laminin have been widely used in cell culture, and also with hESC-RPE cells. Klimanskaya and co-workers have utilized fibronectin and laminin coatings (Klimanskaya et al. 2004; Klimanskaya 2006) and Idelson and co-workers (2009) have successfully utilized laminin coatings in hESC-RPE cell culture. However, these results are not comparable to this study since MEFs, FBS (Klimanskaya et al. 2004; Klimanskaya 2006), and EBs (Idelson et al. 2009) were used. Fibronectin and laminin have also supported human RPE cell attachment (Tezel & Del Priore 1996; Ho & Del Priore 1997). Vitronectin have supported human RPE cell attachment (Ho & Del Priore 1997) and hESC attachment (Braam et al. 2008). On the other hand, hESCs have attached poorly to nidogen+laminin when using defined medium supplements (Braam et al. 2008). It could have been thought that the cell attachment would be better to for example CELLstart™/fibronectin than to CELLstart™ alone, but any difference could not be noticed. Probably the basic coating materials played the crucial role in the cell attachment, so the impact of the additional proteins remained minor. It was also interesting that the cell attachment to collagen IV/CELLstart™ was not better than the attachment to CELLstart™ or collagen IV alone. Nevertheless, it was rather difficult to predict how the proteins of the coating materials would interact if they were mixed. In addition, the composition of CELLstart™ is unclear. In a way it was a desired result that the use of additional proteins or two basic coating materials did not enhance the cell attachment, since the use of additional proteins with the basic coating materials or mixing two basic coating materials was more laborious and time-consuming compared

to using only one basic coating material. In addition, the use of additional proteins or multiple basic coating materials would mean additional costs.

Despite the desired result that additional proteins with basic materials were not superior to basic materials alone, there is an uncertainty concerning the results of the second coating protocol. It is possible that in the second coating protocol (coating first the basic material and then the protein on the basic material) the protein may have diluted the basic material. It was not examined that both coatings were in the wells before the cell passage. The presence of both coating layers such be verified in order to the results of the second coating protocol would be reliable.

Cell pigmentation was noticed on CELLstart™, collagen IV, and VitroCol™ coatings, and therefore this method did not demonstrate differences between these three materials. The pigmented character of RPE cells enables a simple and rapid method of noticing probable differentiation from hESCs. Generally hESC-derived pigmented cells are characterized for instance by immunocytochemistry and q-RT-PCR to verify the differentiation towards RPE cells. Recent publications have published figures of differentiating hESC clusters to show pigmentation (Klimanskaya et al. 2004; Klimanskaya 2006; Vugler et al. 2008; Idelson et al. 2009). In these publications, differentiating hESCs have been cultured as EBs.

Immunocytochemistry did not verify any remarkable differences in hESC differentiation towards RPE cells on tested three coating materials. Some of the stained samples were on many layers, so single cells were difficult to distinguish. However, the cells cultured on CELLstart™, collagen IV, and VitroCol™ for 21 days seemed to express MITF, RAX, and PAX6. The expressions of MITF seemed to be minor. This was expected since it is probable that the cells had not had sufficient time to differentiate. Regardless, the immunocytochemical stainings suggest that there where no remarkable differences in the differentiation on CELLstart™, collagen IV, or VitroCol™ coatings, but the results are not completely reliable due to unclear pictures. A confocal microscope would have given pictures of better quality. On the other hand, other researchers have published clear immunocytochemistry pictures from hESC-RPE cells with a phase contrast microscope. Optimized antibody dilutions with a monolayer of hESC-RPE cells would enable reliable immunocytochemistry pictures with a phase contrast microscope.

q-RT-PCR results did not give consistent results in regard to the differentiation either. When analyzing the q-RT-PCR results, the gene expression levels were examined. MITF is expressed in mature RPE cells so its expression should increase as the experiments proceed. It takes about four to eight weeks of hESCs to differentiate to RPE cells (Idelson et al. 2009; personal communication, Ophthalmology Group/Regea). This experiment lasted only for 21 days so high MITF expressions could not be expected. On the other hand RAX, PAX6, and OTX2 are neural progenitor genes so their expressions can be high at the beginning of the experiment, but they should decrease as the cells differentiate to RPE cells. SIX3 and CHX10 are photoreceptor

progenitor genes, so their expression levels should be low in all time points, but at least at the end of the experiment.

On day 21, the expressions of RAX, PAX6, OTX2, SIX3, and CHX10 were significantly lower in Regea 06/040 cells cultured on collagen IV or VitroCol™ than on CELLstart™. This suggests that Regea 06/040 cells would differentiate to RPE cells better on collagen IV or VitroCol™ coatings than on CELLstart™. The differences in MITF expressions were not significant on day 21. Nevertheless, this can be due to the fact that the cells had not had sufficient time to differentiate. The same as with Regea 06/040 cells, the expression level differences where not consistent for Regea 08/013 cells. On day 14, the expression of MITF was significantly lower in Regea 08/013 cells cultured on collagen IV or on VitroCol™ than on CELLstart™. This suggests that Regea 08/013 cells would differentiate to RPE cells better on CELLstart™ than on collagen IV or VitroCol™ coatings. However, if this suggestion was true, the expression differences should have strengthened on day 21, which did not occur.

It was slightly surprising that CELLstart™ coating did not seem to be remarkable worse than collagen IV or VitroCol™ at enhancing RPE differentiation. After all, according to the manufacturer CELLstart™ should support the hESC expansion of undifferentiated colonies. Therefore it could have been assumed that CELLstart™ would not enhance the differentiation. In addition, this hypothesis is supported by the fact that collagen IV and collagen I (the main component in VitroCol™) have been successfully used in RPE differentiation (Klimanskaya et al. 2004), although in different culture conditions.

Significant differences in the q-RT-PCR results were so minor that according to those results none of CELLstart™, collagen IV, or VitroCol™ coatings seems to be superior in differentiating hESCs towards RPE cells. In addition, multiple incongruities existed between biological replicates. This implies that the samples were heterogeneous, and the differentiation was rather random. Diverse amounts and qualities of the cells could have been passaged to the wells. There should have been several biological replicates so that the randomness would not have affected the results. Later time points would have also given more information. However, it was challenging to lengthen the cell culture for several weeks because the cells and the colonies started to detach from the wells. Even some cavities appeared on some colonies at the end of the experiment. It is possible that the coating material started to detach as the experiment proceeded, which assisted the cell detachment. On the other hand, it is possible that the coating material remained attached, and only the cells started to detach. If the cell culture should be continued for a longer time, some other coating materials or concentrations could also be tested.

Coating materials can also be evaluated on the basis of their easiness and price. The use of VitroCol™ was rather laborious because it had to be neutralized before coating. In addition, VitroCol™ differed from other tested coating materials since it gelled. Formed gel was thickish, and it complicated the analysis of the samples and the pictures. By contrast, the use of CELLstart™ and collagen IV was easy. There are also

great differences in prices. CELLstart™ is four times more expensive and VitroCol™ is 19 times more expensive than collagen IV per well. Coating materials are largely consumed, so these kinds of price differences are remarkable with time.

Altogether, CELLstart™, collagen IV, and VitroCol™ seem to support the attachment and differentiation of hESCs differentiating towards RPE cells equally well with both two tested cell lines (Regea 06/040 and 08/013). There where no remarkable differences between tested cell lines, but the coating materials must be tested with all used cell lines in the future. Due to the small sample size, q-RT-PCR results seemed partly contradictory. However, if one of the tested coating materials would have been superior to others, it would have come out in immunocytochemistry and q-RT-PCR results. Since these tested three coating materials seem to support the cell attachment and differentiation of the cells equally well, but collagen IV is the cheapest and easy to use, it can be recommended as a coating material for hESCs differentiating towards RPE cells.

Nevertheless, tested coating materials did not seem to be very effective in RPE differentiation. The role of growth factors or other supplements in RPE differentiation should be largely studied. For instance, Idelson and co-workers have in a recent study demonstrated that nicotinamide and Activin A can promote the differentiation of hESCs towards RPE cells under defined culture conditions (Idelson et al. 2009). The effects of a combination of examined supplements and collagen IV coating on hESC-RPE cell culture could be studied in the future. Also, a culture medium for hESC-RPE cells without animal-derived serum replacement AlbuMAX should be developed. A xeno-free culture medium would enable optimal culture conditions for hESC-RPE cells.

5.2. Confocal microscopy wells

The aim of this second study was to evaluate the attachment of hESC-RPE cells to commercial Ibidi confocal microscopy wells. As the coating material study showed, it is difficult to distinguish single cells under a phase contrast microscope with fluorescence optics if the sample is on layers. A confocal microscope could solve this problem since it can collect serial optical sections from thick samples. Nevertheless, traditional cell culture plates are not suitable for confocal microscopy. Special confocal microscopy wells have to be tested to find the ones that support the cell attachment.

At first, cell attachment to commercially coated confocal wells was tested in an experiment which lasted for 21 days. hESCs (Regea 06/040 and Regea 08/013) attached well to collagen IV Ibidi wells, but the attachment was poor to ibiTreat and poly-L-lysine Ibidi wells. Pigment formation was noticed in collagen IV Ibidi wells.

The results of this first experiment were quite expected. As well as in the coating material study, cells attached well to collagen IV coated wells throughout the experiment, but poorly to poly-L-lysine coated wells. On the other hand, it is difficult to evaluate the reasons why the cells did not attach to ibiTreat Ibidi wells since the composition of ibiTreat is unknown. There were some remarkable variations between

biological replicates, which is probably due to varieties in the amounts of passaged cells per well. However, on the whole the differences were clear. In addition to the cell attachment, collagen IV Ibidi wells seemed to support hESC differentiation towards RPE cells since pigment formation was noticed. Therefore collagen IV Ibidi wells can be recommended for confocal microscopy for hESCs differentiating towards RPE cells.

When using collagen IV Ibidi wells, attention must be paid to few things. In this experiment, the cells and the colonies started to detach strongly around day 20. Therefore, these wells are not suitable for the long term cell culture of hESC-RPE cells. The size of the wells causes also restrictions. The volume of the wells is very small, and thus the amount of culture medium and passaged cells is limited.

Secondly, the cell attachment to self-coated confocal wells was tested. Uncoated Ibidi wells were coated with the best coating material from commercial Ibidi wells, collagen IV, and compared to self-coated CELLstart™ Ibidi wells for 7-21 days. Mechanically cut RPE cell clusters (differentiated from Regea 08/023) had poor attachment to self-coated collagen IV and CELLstart™ Ibidi wells. On the contrary, enzymatically degraded RPE cells (differentiated from Regea 08/013) attached well to both tested self-coated Ibidi wells. As the experiment proceeded, the attached cells formed colonies in self-coated collagen IV Ibidi wells, unlike in self-coated CELLstart™ Ibidi wells. The double concentrations of collagen and CELLstart™ coatings were also tested, but they were not superior to normal concentrations in regard to the cell (differentiated from Regea 08/023) attachment.

It was unexpected that the mechanically cut small RPE cell clusters did not attach to self-coated collagen IV Ibidi wells since hESCs in RPE DM- medium had attached well to commercially coated collagen IV Ibidi wells. hESCs usually have even worse attachment ability than the differentiated cells. It is possible that the poor attachment of RPE cell clusters is due to the cluster form. Big cell clusters may have difficulties in utilizing adhesion proteins, and therefore have poor attachment ability. On the other hand, mature hESC-RPE cell clusters have been successfully plated for instance on gelatin (Lund et al. 2006) and Matrigel™ (Vugler et al. 2008; Carr et al. 2009). However, the culture conditions and possibly the cluster size were different in the preceding studies compared to this one. On the basis of the results of the coating material study, it was not surprising that the cell attachment was nearly equally good to self-coated CELLstart™ Ibidi wells than to self-coated collagen IV Ibidi wells. On the other hand, mature hESC-RPE cells were used in this experiment, unlike in the coating material study, so the attachment ability could have been different. The interactions between the cells and the coating materials are so complicated and still partly unknown that predictions are often challenging to make.

It was interesting that the enzymatically degraded RPE cells attached remarkably better to self-coated Ibidi wells than the mechanically cut RPE cell clusters. This can not be explained with cell lines because Regea 08/023 -derived RPE cells were used in both degradation methods. Enzymatic degradation with trypsin did not degrade RPE cell clusters to single cells, but only few RPE cells remained together. Probably the adhesion

proteins could attach the small cell groups better to the coating materials than they could attach the cell clusters. However, enzymatic degradation is not optimal for the cells because the exposure to enzymes may cause genetic abnormalities (Buzzard et al. 2004; Mitalipova et al. 2005), and used enzymes are often xeno-products. In the future, it would be important to find culture conditions that would support mechanically degraded hESC-RPE cell attachment, since mechanical degradation is gentler to the cells than enzymatic degradation. RPE cell clusters should be degraded to extremely small pieces.

It was also interesting that the attached enzymatically degraded RPE cells formed colonies in self-coated collagen IV Ibidi wells, unlike in self-coated CELLstart™ Ibidi wells. In the coating material study, hESCs in RPE DM- medium attached and formed colonies equally well on these coating materials. Therefore it seems that the collagen IV coating supports the growth and colony formation of mature RPE cells better than CELLstart™ coating. On the other hand, the formed colonies in self-coated collagen IV Ibidi wells contained also other cells than RPE cells. Unfortunately this experiment had to be finished for external reasons. It would have been interesting to observe if the attached cell clusters in self-coated CELLstart™ Ibidi wells had grown and formed colonies, and if there had been more RPE cells than on the colonies in self-coated collagen IV Ibidi wells. In the future, longer-term experiments should be performed to study these things. In addition, it would be interesting to see when the attached cells and colonies would detach from these self-coated Ibidi wells. For long-term cell culture in confocal microscopy wells, it would be important to find the culture conditions where the detachment of the cells and the colonies would occur far later than on day 20, when the detachment occurred in commercially coated collagen IV Ibidi wells.

5.3. Cell culture in inserts

The aim of this third study was to evaluate BD BioCoat™ collagen IV cell culture inserts with hESC-RPE cells, and the effect of bFGF in the culture medium. Mechanically cut hESC-RPE cell clusters (differentiated from Regea 08/013) were passaged into the inserts containing either RPE DM-, RPE DM- +5 ng/ml bFGF, or RPE DM- +10 ng/ml bFGF medium, and the attachment and growth of RPE cell clusters were observed for 17 days. The cell clusters attached and grew well in all three tested culture conditions, and there were no remarkable differences. Visually observed, bFGF did not seem to trandifferentiate hESC-RPE cells into neural retina cells or neurons.

bFGF is generally used in hESC medium to promote self-renewal and pluripotency, whereas bFGF is generally removed from RPE medium to promote differentiation. On the contrary, Klimanskaya (2006) have reported that after the isolation of hESC-RPE cells, bFGF accelerates the cell proliferation and reacquisition of the RPE morphology. This study showed that the presence of bFGF in the cell culture of mature RPE cells did not have a remarkable effect on the cell attachment. However, it was interesting to notice that few RPE cell clusters had probably divided in the inserts with medium

containing bFGF, which would be parallel to Klimanskaya's results. Further studies should be carried out to test if bFGF really promotes RPE cell cluster distribution.

bFGF have been reported to induce the transdifferentiation of RPE cells into neural retina (Galy et al. 2002) and neuronal progenitors (Opas & Dziak 1994), but this was not noticed in this study by visual observation. Possible transdifferentiation could be studied thoroughly for instance by q-RT-PCR.

Carr and co-workers (2009) and Nistor and co-workers (2010) have used inserts in their RPE cell culture studies, but the culture conditions in their studies have been different from those of this study. In Carr and co-workers' experiments, the phagocytosis of POS from the human retina was studied. hESC-RPE cells were cultured on Matrigel™ coated filters and exposed to a photoreceptor cell layer of the retina tissue explants. Inserts were used to hold the RPE cell layer and the POSs together. In Nistor and co-workers' studies, hESC-derived neural retinal progenitors were cultured in a matrix on top of hESC-RPE cells in collagen/laminin or poly-L-lysine/laminin coated inserts in order to develop 3D tissue constructs. Osmolarity gradient was maintained in the cell culture insert system. Nistor and co-workers discovered that small size of insert pores (diameter 0.2-1.0 μm) did not allow for sufficient media exchange, whereas larger pores (3 μm) allowed for nutrient exchange proved by healthy 3D tissue constructs. Likewise, the small size of cell culture inserts (diameter 5 mm) did not allow for sufficient media supply, whereas larger inserts (up to 3 cm) resulted in tissue survival.

In this study, the diameter of the pore was 1.0 μm and the diameter of the insert was 6.4 mm, which can be classified as small according to Nistor and co-workers. Therefore, the good growth of hESC-RPE cells in this research project is contradictory to Nistor and co-workers' results. Nevertheless, the results cannot be directly compared since the culture conditions were different. Carr and co-workers' and Nistor and co-workers' studies showed that cell culture inserts can be utilized in co-culture experiments. This research project showed that the inserts can also be used to grow hESC-RPE cells without co-culture. Inserts provide culture environment resembling *in vivo* state due to the pores and free diffusion of nutrients.

Collagen IV in BD BioCoat™ inserts have been extracted from mouse, and therefore culture conditions in the inserts are not xeno-free. Consequently, the cells cultured in these inserts are not optimal for clinical applications. Nevertheless, the inserts supported the attachment and growth of hESC-RPE cell clusters effectively, and the cells can be utilized in other research purposes. It is a remarkable result that the attachment was good in serum-free culture conditions. In longer-term experiments, hESC-RPE cell clusters have grown so well that the inserts have become confluent.

6. CONCLUSIONS

The aim of this study was to evaluate the cell attachment and differentiation of hESC-RPE cells on different coating materials and culture conditions. hESCs were derived and maintained without serum and animal feeder cells.

In the first part of this study, the attachment and differentiation of hESCs differentiating towards RPE cells on several xeno-free coating materials and different material combinations were tested. CELLstart™, collagen IV, and VitroCol™ were the best coating materials to support the cell attachment and differentiation. Nevertheless, collagen IV is clearly cheaper than CELLstart™ or VitroCol™, and easy to use, so it can be recommended as a coating material for hESCs differentiating to RPE cells. To my knowledge no study of hESC differentiation towards RPE cells with a xeno-free coating material and a serum-free culture medium in adherent cell culture has been published, thus this result of good cell attachment without FBS is remarkable. Due to this study, Ophthalmology group in Regea uses collagen IV as a coating material for hESCs differentiating to RPE cells. In the future, the effects of growth factors and other supplements on RPE differentiation could be studied.

In the second part of this study, confocal microscopy wells were tested. hESCs differentiating to RPE cells and enzymatically degraded hESC-RPE cells attached and grew well in collagen IV coated Ibidi wells. Enzymatically degraded hESC-RPE cells attached also well to CELLstart™ coating, but they did not form colonies as they did on collagen IV coating. Longer-term effects of collagen IV and CELLstart™ coated Ibidi wells on hESC-RPE cell attachment and differentiation should be researched in further studies.

In the third part of this study, the attachment of hESC-RPE cells to BD BioCoat™ collagen IV cell culture inserts, and the effect of bFGF in the culture medium were evaluated. Mechanically cut hESC-RPE cell clusters attached and grew well in the inserts with or without bFGF. bFGF did not seem to transdifferentiate hESC-RPE cells into neural retina cells or neurons. It was a remarkable result that hESC-RPE cells attached well to the inserts without FBS. On the contrary, BD BioCoat™ collagen IV cell culture inserts are not xeno-free, and therefore the cells cultured in these inserts are not optimal for clinical applications. Nevertheless, these inserts can be used in research purposes, and due to this study they are in use in Ophthalmology group in Regea. In the future, the ability of bFGF to promote RPE cell cluster distribution and to transdifferentiate RPE cells could be further studied.

These results of good hESC-RPE cell attachment in serum-free culture conditions are important steps in RPE research. Also, this study has opened doors for further

studies. In addition to the research subject proposals mentioned above, a culture medium for hESC-RPE cells without animal-derived serum replacement AlbuMAX should be developed. A xeno-free culture medium would enable optimal culture conditions for hESC-RPE cells.

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